Mycobacterium tuberculosis induced transcription in macrophages: the role of TPL2/ERK signalling in the negative regulation of type I interferon production and implications for control of tuberculosis

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I, John Benson Ewbank, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis

Abstract

Mycobacterium tuberculosis is an important global cause of mortality and morbidity. The major host cell of *Mycobacterium tuberculosis* is the macrophage, and *Mycobacterium tuberculosis* is able to subvert the macrophage response in order to survive and replicate. The majority of infected individuals mount an immune response capable of controlling *Mycobacterium tuberculosis* infection. This requires the cytokines IL-12, TNFα, IL-1 and IFNγ, which promote eradication or control of infection. However, other immune factors, including IL-10 and type I IFN, can inhibit this protective response.

In this study we have used microarray analysis to study the temporal response of macrophages to *Mycobacterium tuberculosis* infection, in an unbiased fashion. In response to *Mycobacterium tuberculosis* infection, macrophages produced cytokines and chemokines, upregulated genes involved with major histocompatability class I antigen presentation, activated both pro- and anti-apoptotic genes and downregulated many genes involved in cell-division and metabolism. We also observed the early induction of genes regulated by the extracellular-regulated kinase (ERK) MAP kinase pathway, and the upregulation of genes known to be induced by type I IFN, leading us to further investigate the role of these pathways in the macrophage response to *Mycobacterium tuberculosis*. Both pathways were found to regulate the production of protective and detrimental cytokines in macrophages in response to *Mycobacterium tuberculosis* infection. In addition, microarray analysis found that these pathways controlled the transcription of numerous genes in response to *Mycobacterium tuberculosis* infection. Finally, type I IFN was found to inhibit the macrophage response to IFNγ, including IFNγ-mediated killing of *Mycobacterium tuberculosis* in macrophages, a crucial step in the control of *Mycobacterium*

tuberculosis infection *in vivo*. We have therefore identified important regulatory mechanisms in macrophages, which are likely have an important role during *Mycobacterium tuberculosis* infection *in vivo*.

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List of Abbreviations

AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BCG	Bacille Calmette-Guerin
CD	Cluster of differentiation
CFU	Colony forming unit
CpG	Cytosine-phosphodiester-guanine
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-related kinase
FCS	Fetal calf serum
HIV	Human immunodeficiency virus
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
IL-	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
MDR-TB	Multi-drug resistant tuberculosis
MHC	Major histocompatability complex
MOI	Multiplicity of infection
Mtb	*Mycobacterium tuberculosis*
MRC NIMR	Medical Research Council National Institute for Medical Research
NK	Natural Killer
NOD	Nucleotide-binding oligomerisation domain
NLR	NOD-like receptor
OADC	Oleic acid-albumin dextrose catalase
OD	Optical density

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
TB	Tuberculosis
Th	T helper cell
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPL2	Tumour progression locus 2
TRIF	TIR domain containing adaptor protein inducing IFN
WT	Wild-type
XDR-TB	Extensively drug resistant tuberculosis

Chapter 1. Introduction

1.1. Tuberculosis and *Mycobacterium tuberculosis*

1.1.1. Tuberculosis: a major global health problem

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*), has been a cause of mortality in human beings for thousands of years (Salo *et al*, 1994). However, despite great progress in the understanding of TB since the discovery of *Mtb* by Robert Koch (Koch, 1882) there were 8.8 million new TB cases in 2010 and 1.45 million deaths (WHO, 2011). The majority of the global TB burden is in the developing world, particularly in Asia and Sub-Saharan Africa (WHO, 2011).

Following the discovery of effective antibiotics for the treatment of TB, such as Streptomycin, it was thought that TB could be controlled and potentially eradicated. However, there has been a steady increase in the global burden of TB over the last 20 years, for three important reasons (Dye and Williams, 2010). First, although antibiotics are a highly efficacious and cost-effective means of treating TB, the rise in multi-drug resistant (MDR) strains of *Mtb* has hampered these efforts, and threatens to become a major health problem (Jassal and Bishai, 2009). Essentially untreatable extensively drug-resistant (XDR) strains are now being reported, which are associated with high mortality in HIV patients (Jassal and Bishai, 2009). Second, the human immunodeficiency virus (HIV) epidemic has fuelled the global rise in TB cases. HIV infects and kills $CD4^+$ T cells, leading to a greatly increased risk of developing TB (Kwan and Ernst, 2012). Almost a quarter of TB deaths in 2010 occurred in HIV positive individuals, and TB is the biggest killer of patients with acquired immune deficiency syndrome (AIDS) (WHO, 2011). Finally, the lack of an effective vaccine against *Mtb* is a major problem (Kaufmann, 2012). Although the

current vaccine, *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), can protect children against rare forms of disseminated TB (Trunz *et al*, 2006), its efficacy in protecting adults from pulmonary TB, the most common form of the disease, is highly variable (Colditz *et al*, 1994; Fine, 1995).

1.1.2. The outcome of infection with *Mtb*

TB is predominantly a lung infection, although it can disseminate to other organs (Lawn and Zumla, 2011). *Mtb* is spread from person to person via the aerosol route, in water droplets containing infectious bacteria which penetrate into the alveoli and initiate infection (Lawn and Zumla, 2011). There are several possible outcomes for an individual following infection with *Mtb* (Flynn and Chan, 2001b) which are summarised in Figure 1.1.

A small percentage of infected individuals are unable to control the initial infection, and develop acute active disease, characterised by fever, weight loss, night sweats and a persistent cough (Flynn and Chan, 2001b; Lawn and Zumla, 2011). However, the majority of infected individuals mount an immune response capable of controlling, but not clearing, the infection (Barry *et al*, 2009). This state, known as latency, is defined by evidence of an adaptive immune response to *Mtb* antigens, as shown by the tuberculin skin test (TST) to mycobacterial antigens, or the IFNγ release assay (IGRA) to *Mtb*-specific antigens, with an absence of disease symptoms and contagiousness (Flynn and Chan, 2001b). However, it is becoming increasing appreciated that latency as determined by the TST or IGRA is likely to encompass a spectrum of different disease states, ranging from a resolved infection to subclinical disease (Barry *et al*, 2009).

Approximately one-third of the world's population is latently infected with *Mtb*, and around 10% of these will go on develop active TB, often years after the initial infection (Dye *et al*, 1999) (Figure 1.1). The immune system is vital in maintaining latency, and factors that compromise the immune system, such as HIV co-infection or neutralisation of TNFα, dramatically increase the risk of reactivation (Keane *et al*, 2001; Kwan and Ernst, 2012) (Figure 1.1).

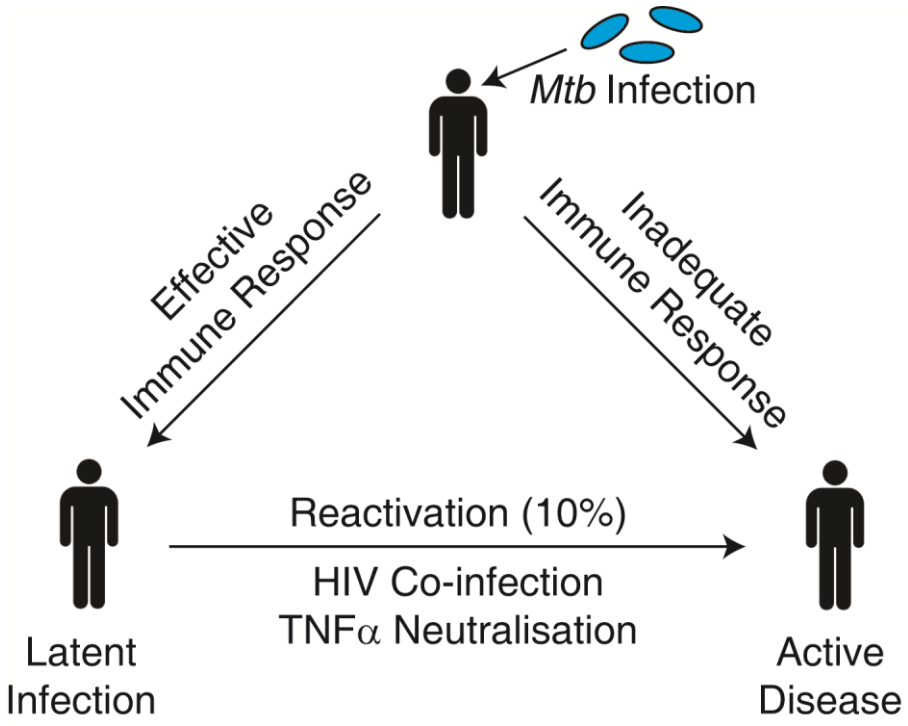

Figure 1.1. The outcome of infection with *Mtb*

1.1.3. Different strains of *Mtb* show increased virulence and may affect disease outcome

One factor that may have an influence on disease outcome is the strain of *Mtb* with which an individual is infected (Nicol and Wilkinson, 2008). Research into *Mtb* genetics has revealed that the *Mtb* complex consists of seven main lineages (two of which include strains of *M. africanum*) which are associated with different

geographical regions (Gagneux and Small, 2007; Hershberg *et al*, 2008). These findings have prompted research to address whether different strains of *Mtb* are associated with changes in virulence, and changes in the immune response, predominantly by comparing different strains in animal models (Hershberg *et al*, 2008) or human and mouse cells (Reed *et al*, 2004; Newton *et al*, 2006). Several strains of *Mtb* have been identified that show increased virulence in animal models compared to reference strains such as H37Rv, and prominent among these are members of the W-Beijing family (Parwati *et al*, 2010). Members of this family such as HN878 are hypervirulent in mouse models (Manca *et al*, 2001) and infection of humans with W-Beijing family members has been associated with an increased likelihood of reactivation (Kong *et al*, 2007). One mechanism behind the increased virulence of W-Beijing strains appears to be an ability to suppress the production of important cytokines from innate immune cells (Reed *et al*, 2004). In addition, other *Mtb* strains not in the W-Beijing lineage can suppress the host immune response; one study isolated an *Mtb* strain from a large outbreak in Leicester, the CH strain, and found that this strain induced higher levels of the immunosuppressive cytokine IL-10, but lower levels of the protective cytokine IL-12p40 (Newton *et al*, 2006). Thus, the outcome of infection with *Mtb* may depend strongly on the virulence of the infecting strain, and how effectively that strain can subvert the immune response.

1.2. Early events following *Mtb* infection

Given the importance of the immune response in controlling *Mtb*, how the immune system responds to *Mtb* infection has been studied extensively in recent years, primarily using animal models. This has shown that the immune response to *Mtb* consists of several key stages, culminating in the control of the infection in the

lung (Figure 1.2). The first stages involve the interactions of *Mtb* with cells of the innate immune system, which is discussed below.

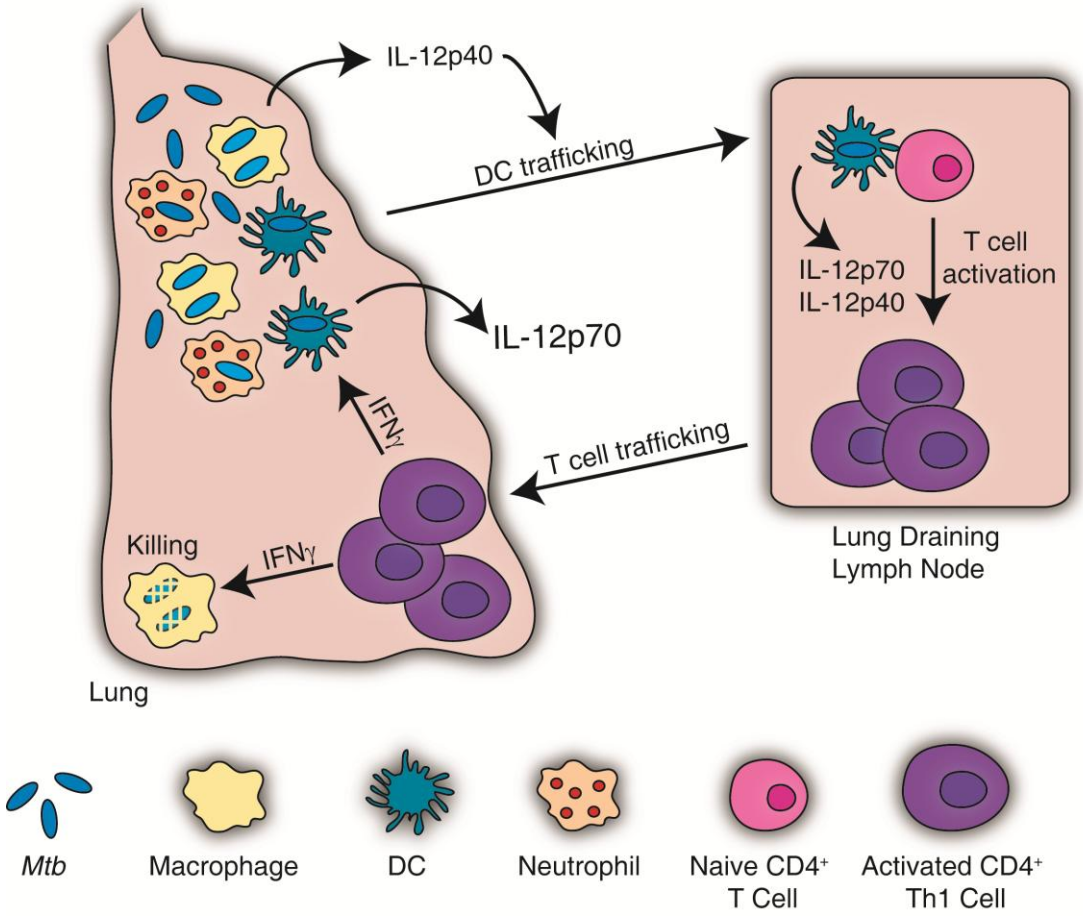

Figure 1.2. An overview of the immune response to *Mtb*.

1.2.1. The innate immune system

The first immune cells encountered by *Mtb* following infection are those of the innate immune system (Figure 1.2). The role of innate immune cells, which include macrophages, dendritic cells (DCs) and neutrophils, is to rapidly detect and eliminate pathogens entering the body, and to activate the adaptive immune response (Medzhitov, 2007). Innate immune cells must therefore be able to respond

specifically to pathogens, such as *Mtb*, as inappropriate responses could lead to host damage or immunosuppression (Smale, 2010).

Specificity is initially achieved through pattern recognition receptors (PRRs), germ-line encoded receptors expressed on immune cells (Janeway and Medzhitov, 2002). PRRs recognise molecular structures that are unique to microorganisms, which are known as pathogen-associated molecular patterns (PAMPs) (Medzhitov, 2007). A classic example of a PAMP is lipopolysaccharide (LPS), a molecule specific to the cell walls of gram-negative bacteria (Kawai and Akira, 2010). Recognition of a PAMP by a PRR on an immune cell stimulates various processes including phagocytosis, production of cytokines and chemokines and induction of antigen presentation (Medzhitov and Horng, 2009).

1.2.2. *Mtb* infects cells of the innate immune system in the lung

It is thought that the first innate immune cells to encounter *Mtb* are lung-resident alveolar macrophages (Flynn and Chan, 2001a). *Mtb* infects alveolar macrophages and although these cells are specialised to destroy foreign material such as bacteria, it has long been recognised that *Mtb* has evolved to avoid these killing mechanisms, and actively replicates within macrophages (Armstrong and Hart, 1971). Replication results in inflammation and the influx of further innate immune cells into the lung, including monocytes, macrophages, DCs and neutrophils (Wolf *et al*, 2007; Skold and Behar, 2008). Although macrophages are thought to be the major infected cell type, experiments with GFP labelled *Mtb* have shown that monocytes, DCs and neutrophils can also become infected (Wolf *et al*, 2007).

Infected neutrophils have also been reported in the lungs of human TB patients (Eum *et al*, 2010).

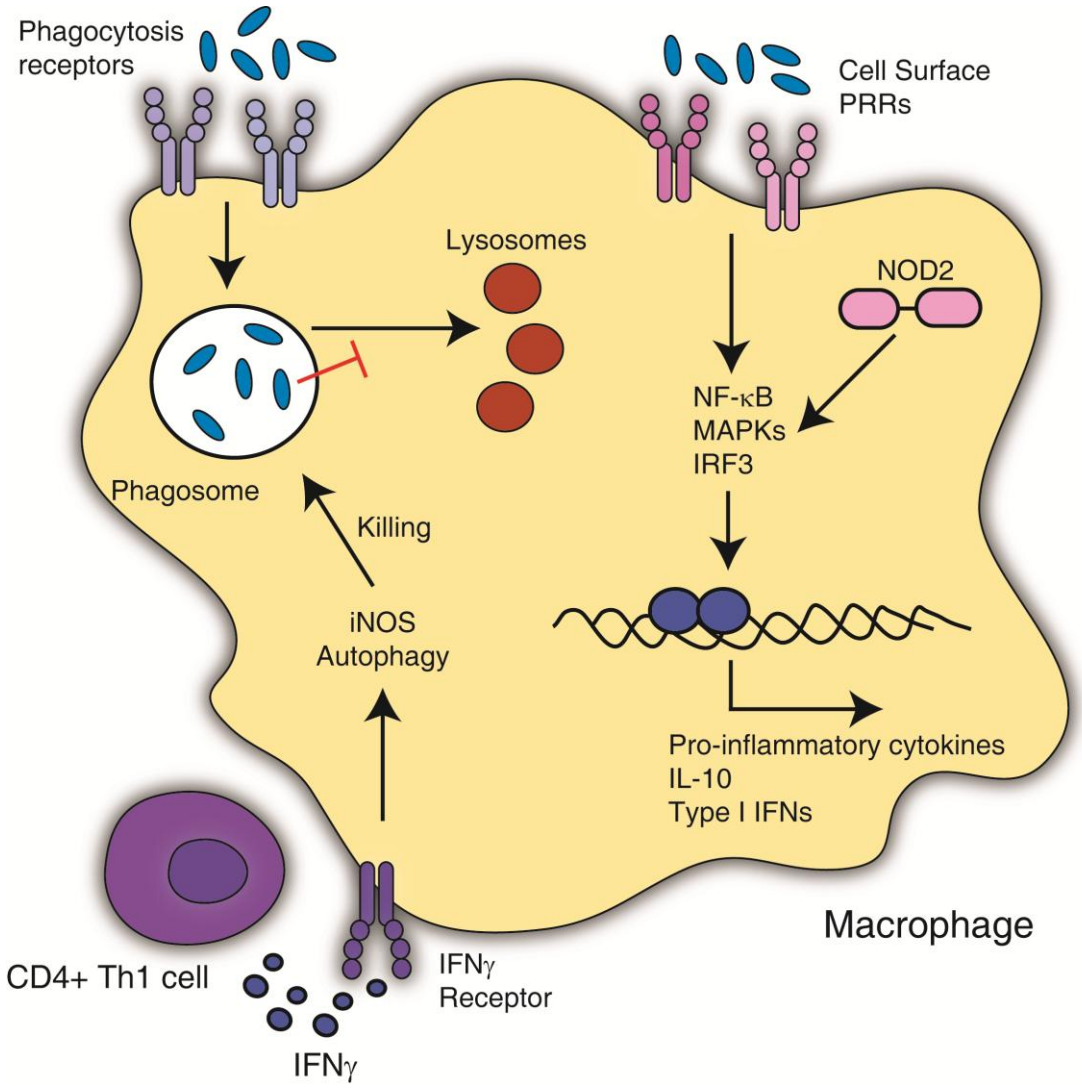

Figure 1.3. An overview of the macrophage response to *Mtb*

1.2.3. The interactions between *Mtb* and macrophages

As the macrophage is one of the major infected cell types during *Mtb* infection, and is thought to mediate *Mtb* killing, the interactions between *Mtb* and macrophages are crucial, and have been extensively studied (Pieters, 2008). These interactions are summarised in Figure 1.3. Several important processes are activated

in macrophages upon encountering *Mtb*, including phagocytosis and the destruction of *Mtb*, and the induction of cytokines downstream of recognition by PRRs (Pieters, 2008; Kleinnijenhuis *et al*, 2011). However, *Mtb* can subvert these processes, allowing it to replicate within macrophages (Pieters, 2008) (Figure 1.3).

1.2.3.1. *Mtb* can avoid macrophage killing mechanisms

A key function of macrophages is to take up and destroy foreign material, including bacteria, through a process called phagocytosis. Phagocytosis of *Mtb* into macrophages and other innate immune cells is stimulated through various receptors on the macrophage surface, including the mannose receptor (MR), dendritic cell-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) (Tailleux *et al*, 2003), Fc receptors and complement receptors (Ernst, 1998; Pieters, 2001). Phagocytosis results in *Mtb* being contained within an intracellular vesicle known as the phagosome (Ernst, 1998) (Figure 1.3).

Under normal circumstances, the phagosome enters the endosomal pathway, culminating in fusion with a lysosome, a process known as phagosome-lysosome fusion (Vergne *et al*, 2004). Lysosomes contain potent hydrolytic enzymes, and are highly acidic (pH 4.5-5), and this destroys phagocytosed material and generates antigens that are loaded onto major histocompatability (MHC) class II molecules and presented on the cell surface (Pieters, 2008). However, *Mtb* has a number of strategies for blocking phagosome-lysosome fusion, involving several different *Mtb* virulence factors (Pieters, 2008). As a result, phagosomes containing *Mtb* fail to acidify or to acquire mature lysosomal hydrolases (Sturgill-Koszycki *et al*, 1994; Russell, 2001). *Mtb* blocks the recruitment of phosphatidylinositol 3-phosphate (PI3P) to the phagosome, a process that is essential for phagosome maturation (Fratti

et al, 2003). This is achieved by the inhibition of the enzyme that generates PI3P, hVP34, and by the breakdown of PI3P through the activity of the *Mtb* encoded phosphatase SapM (Pieters, 2008). The mycobacterial kinase protein kinase G (PknG) is also required for the prevention of phagosome-lysosome fusion (Walburger *et al*, 2004), although the mechanism for this is currently unknown. Finally, *Mtb* actively induces the recruitment of Coronin-1 to the phagosome, which leads to the activation of the enzyme calcineurin, resulting in prevention of phagosome-lysosome fusion (Jayachandran *et al*, 2007). As a result of these virulence strategies, macrophages require activation by IFNγ, following the initiation of the adaptive immune response, in order to activate pathways leading to the destruction of intracellular *Mtb* (Pieters, 2008) (Figure 1.3).

1.2.3.2. Recognition of *Mtb* through PRRs on innate immune cells

An important response of macrophages to *Mtb* infection is to produce cytokines which lead to the recruitment and activation of further immune cells to the lung (Kleinnijenhuis *et al*, 2011). *Mtb* is known to possess several different PAMPs, which are recognised by several different types of PRRs on innate cells such as macrophages and DCs (Figure 1.3).

1.2.3.2.1. Toll-like receptors

A major family of PRRs implicated in the recognition of *Mtb* are the Toll-like receptors (TLRs), a family of type I transmembrane proteins expressed in innate immune cells, that recognise diverse components of microorganisms (Medzhitov, 2007). Two major pathways are activated downstream of TLR signalling depending on the adaptor protein used (Kawai and Akira, 2010). All TLRs except TLR3 signal

through the myeloid differentiation primary response gene 88 (MyD88) adaptor protein, activating the MyD88-dependent pathway (Kawai and Akira, 2010). The cytokines IL-1 and IL-18 also signal through MyD88 (Dinarello, 2009). This pathway culminates in activation of the transcription factor NF-κB and the mitogen activated protein (MAP) kinases, leading to the induction of many genes, including pro-inflammatory cytokines (Kawai and Akira, 2010). TLR2 and TLR4 also require the adaptor toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) to activate the MyD88 pathway (Horng *et al*, 2002; Yamamoto *et al*, 2002). TLR3 and TLR4 activate the adaptor molecule TIR-domain-containing adapter-inducing interferon-β (TRIF), and the TRIF dependent pathway. The TRIF-dependent pathway is primarily involved in the induction of type I interferon (IFN) through activating the transcription factors IRF3 and IRF7 (Kawai and Akira, 2010).

The main TLRs involved in the recognition of *Mtb* are TLR2, TLR4 and TLR9 (Kleinnijenhuis *et al*, 2011). TLR2, in complex with either TLR1 or TLR6, has been shown to recognise various cell wall components of *Mtb* such as lipoarabinomannan (LAM), lipomannan (LM), and the 38-kDa and 19-kDa glycoproteins (Kleinnijenhuis *et al*, 2011). TLR9 recognises unmethylated CpG motifs in mycobacterial DNA (Bafica *et al*, 2005). Finally, *Mtb* heat-shock proteins have been suggested to activate TLR4 (Bulut *et al*, 2005). Macrophages and DCs deficient in TLR2, TLR4 or TLR9 showed impaired production of the cytokines TNFα, IL-12, IL-1 and IL-10 in response to *Mtb* infection (Jang *et al*, 2004; Bafica *et al*, 2005; Pompei *et al*, 2007; Holscher *et al*, 2008; Kleinnijenhuis *et al*, 2009).

However, despite a clear role for TLR signalling *in vitro*, the relevance of TLRs in the response to *Mtb in vivo* has been controversial (Korbel *et al*, 2008;

Reiling *et al*, 2008). Various groups have infected TLR2, TLR4 and TLR9 knockout mice, as well as double and triple knockouts, but results have been contradictory, with some observing a severe phenotype with single knockout mice (Abel *et al*, 2002; Reiling *et al*, 2002; Drennan *et al*, 2004), whilst others find no effect even with mice triply deficient in TLR2, TLR4 and TLR9 (Holscher *et al*, 2008). These discrepancies are likely to result from differences in the strain and dose of *Mtb* used. For example, it has been suggested that TLRs play a more important role in response to high-dose *Mtb* infection (Reiling *et al*, 2002).

However, MyD88$^{-/-}$ mice, which are unable to signal through any TLR apart from TLR3, have consistently been observed to succumb rapidly to *Mtb* infection (Fremond *et al*, 2004; Scanga *et al*, 2004; Fremond *et al*, 2007; Holscher *et al*, 2008). The explanation for this severe phenotype is now thought to be due to loss of IL-1 signalling, rather than TLR signalling (Korbel *et al*, 2008; Reiling *et al*, 2008), as IL-1 signals through MyD88 (Sims and Smith, 2010). The role of IL-1 in the immune response to *Mtb* is discussed below.

1.2.3.2.2. Dectin-1

Non-TLR PRRs can also recognise *Mtb* components and mediate lead to cytokine production in innate immune cells (Kleinnijenhuis *et al*, 2011). One of these is dectin-1, a receptor in the C-type lectin family expressed on monocytes, macrophages, DCs and a subset of T cells (Brown, 2006). Dectin-1 recognises β-glucans, carbohydrate polymers found in fungal cell walls (Brown *et al*, 2002), and activates a Syk-CARD9-ERK dependent pathway leading to induction of TNFα, IL-2 and IL-10 (Brown *et al*, 2003; LeibundGut-Landmann *et al*, 2007; Slack *et al*, 2007; Zhang *et al*, 2009). Dectin-1 was shown to be required for optimal IL-12

production from splenic DCs infected with *Mtb* (Rothfuchs *et al*, 2007). However, dectin-1 appears to play a minimal role in the *in vivo* response to *Mtb*, as dectin-1 deficient mice showed similar survival, bacterial loads and cytokine production following *Mtb* infection, compared to WT controls (Marakalala *et al*, 2011).

1.2.3.2.3. DC-SIGN

DC-SIGN, expressed predominantly on DCs, is another PRR in the C-type lectin family implicated in the recognition of *Mtb* (Kleinnijenhuis *et al*, 2011). DC-SIGN is an important phagocytosis receptor for *Mtb* on human DCs and alveolar macrophages (Tailleux *et al*, 2003; Tailleux *et al*, 2005). However, it has been suggested that *Mtb* may target DC-SIGN in order to promote the production of IL-10 from DCs, and suppress DC function (Geijtenbeek and van Kooyk, 2003), through a pathway involving Raf-1 and the acetylation of NF-κB (Gringhuis *et al*, 2007; Gringhuis *et al*, 2009). However, this potentially negative role of DC-SIGN is in conflict with the fact that mutations that increase the expression of DC-SIGN in humans are associated with protection against TB (Barreiro *et al*, 2006), and that in mice, deletion of a homolog of DC-SIGN, SIGNR3, leads to increased susceptibility to *Mtb* infection (Tanne *et al*, 2009).

1.2.3.2.2. NOD2

Nucleotide-binding oligomerisation domain-containing protein 2 (NOD2) is a member of the NOD-like receptor (NLR) family of PRRs and is expressed in the cytosol of a variety of immune cells (Franchi *et al*, 2009). NOD2 recognises the muramyl dipeptide (MDP) component of peptidoglycan, a structure found in the bacterial cell wall (Franchi *et al*, 2009). NOD2 was shown to be required for optimal

TNFα production from murine peritoneal macrophages infected with *Mtb* (Ferwerda *et al*, 2005; Yang *et al*, 2007). NOD2 recognition has subsequently been shown to mediate induction of IL-1 (Kleinnijenhuis *et al*, 2009) and type I IFNs (Pandey *et al*, 2009) in *Mtb* infected macrophages. This recognition appears to be important for the immune response, as NOD2 deficient mice have impaired control of *Mtb* infection (Divangahi *et al*, 2008).

1.2.4. Dendritic cells initiate the adaptive immune response to *Mtb*

As the innate immune system is unable to control *Mtb* infection, the adaptive immune system is required to be activated in order mediate protection. The activation of the adaptive immune response, and in particular the activation of T cells, is a crucial stage in the immune response to *Mtb* (North and Jung, 2004; Cooper, 2009) (Figure 1.2). T cells are present in the periphery and lymphoid organs in a naive state; in order to become activated and fight infection they must recognise their specific cognate antigen on the surface of MHC molecules, whilst simultaneously receiving co-stimulatory signals (Banchereau and Steinman, 1998). $CD4^+$ T cells recognise antigens on MHC class II molecules, whereas $CD8^+$ T cells recognise antigen MHC class I molecules (Vyas *et al*, 2008). Different pathways lead to antigen presentation on MHC class I and MHC class II receptors (Vyas *et al*, 2008). Antigens derived from cytosolic pathogens such as viruses and certain bacteria are processed in the cytosol and presented on MHC class I molecules to $CD8^+$ T cells, whereas extracellular and phagosomal pathogens, such as *Mtb*, are largely processed via the endocytic pathway and presented on MHC class II molecules to $CD4^+$ T cells (Vyas *et al*, 2008). However, it is also possible for

antigens in the endocytic pathway to be presented on MHC class I molecules, and *vice versa*, through a process known as cross-presentation, although the mechanisms behind this are not well understood (Vyas *et al*, 2008). In response to recognition of their cognate antigens, T cells produce IL-2, proliferate, acquire effector functions and migrate to the site of infection where they activate other cells of the immune system, in the case of $CD4^+$ T cells, and directly kill infected cells, in the case of $CD8^+$ T cells (Banchereau and Steinman, 1998).

Cells capable of activating naive T cells are known as antigen presenting cells (APCs), and the most efficient of these is the DC (Banchereau and Steinman, 1998). DCs capture antigen at the site of infection, and migrate to the lymphoid organs, where they present the antigen on both MHC class I and class II molecules. DCs also express the necessary costimulatory molecules, such as CD80 and CD86, and are therefore highly effective at activating naive T cells (Banchereau and Steinman, 1998). These abilities of DCs are controlled by recognition of foreign microorganisms through PRRs, with PRR recognition stimulating increased phagocytosis, upregulation of MHC class I and class II antigen presentation components and upregulation of costimulatory molecules and cytokines (Vyas *et al*, 2008). The cytokine IFNγ, which is produced by T cells and NK cells, also stimulates increased antigen presentation in innate immune cells (Boehm *et al*, 1997).

$CD4^+$ T cells are the key effector cell in controlling *Mtb* infection, and the events leading to activation of $CD4^+$ T cells have been extensively studied (Cooper, 2009). Following *Mtb* infection, evidence has consistently shown that DC migration from the lung to the lung-draining lymph nodes (LDLN) is required for the

activation of T cells (Cooper, 2009). It was first shown that live *Mtb* appears in the LDLN between 9 and 11 days post-infection, and that this precedes T cell activation (Chackerian *et al*, 2002). Subsequently, transfer of *Mtb*-specific naive $CD4^+$ T cells confirmed that T cells were first activated in the LDLN, and then migrated to the lungs (Reiley *et al*, 2008; Wolf *et al*, 2008). A study using GFP-tagged *Mtb* showed that the major infected cell type in the LDLN were a population of $CD11c^{hi}$ $CD11b^{hi}$ myeloid DCs, which were also present in the lungs (Wolf *et al*, 2007). This evidence strongly suggests that the T cell response is activated by a population of DCs that migrate from the lung to the LDLN, carrying *Mtb* antigens. Activated T cells then traffic to the infected lung and mediate control of the infection (Cooper, 2009; shown in Figure 1.2).

$CD8^+$ T cells specific to *Mtb* antigens are also activated following infection, and although not as critical for protection as $CD4^+$ T cells, still play a role in limiting bacterial replication (North and Jung, 2004). Activation of $CD8^+$ T cells during *Mtb* infection requires cross-presentation of phagosomal *Mtb* antigens on MHC class I molecules. Two pathways have been proposed for this process (Vyas *et al*, 2008). First, antigens may exit from the phagosome into the cytosol, and subsequently be processed by the proteasome and enter the endoplasmic reticulum (ER) via the TAP transporter, as in classical MHC class I antigen processing (the cytosolic pathway) (Vyas *et al*, 2008). Second, antigens in the phagosome may traffic to a vesicle containing recycled MHC class I molecules, and thus bypass the proteasome and ER (the vesicular pathway) (Vyas *et al*, 2008). Several studies have shown that cross-presentation of a variety of *Mtb* antigens by human DCs requires the proteasome and the TAP transporter, although exactly how *Mtb* antigens exit from the phagosome

remains unclear (Lewinsohn *et al*, 2006; Grotzke *et al*, 2009; Grotzke *et al*, 2010). However, in contrast to these studies, cross-presentation of the 19kDa antigen of *Mtb* was shown to be TAP-independent (Neyrolles *et al*, 2001) and cross-priming of apoptotic vesicles by bystander-DCs was also shown to be proteasome-independent (Schaible *et al*, 2003), supporting a role for the vesicular pathway.

1.2.5. Apoptosis of innate immune cells promotes immunity to *Mtb*

An important response of macrophages and other cells infected with *Mtb* is the induction of cell death via apoptosis (Behar *et al*, 2011). Cell death through apoptosis results in the cell breaking up into small membrane-bound vesicles, and is distinct from necrotic cell death which is characterised by disruption of the plasma membrane (Behar *et al*, 2011). Macrophage death by apoptosis promotes the host response to *Mtb*, first because it reduces the viability of *Mtb* (Oddo *et al*, 1998; Divangahi *et al*, 2009) and second because the apoptotic vesicles contain *Mtb* antigens, which are taken up by DCs and used to cross-prime $CD8^+$ T cells, leading to an enhanced $CD8^+$ T cell response (Schaible *et al*, 2003; Winau *et al*, 2006).

The beneficial effects of apoptosis in the immune response are highlighted by the fact that virulent strains of *Mtb* are able to inhibit apoptosis in macrophages, leading instead to necrosis which promotes inflammation and bacterial dissemination (Chen *et al*, 2006; Gan *et al*, 2008; Divangahi *et al*, 2010). The virulent strain of *Mtb*, H37Rv, has consistently been shown to induce greater levels of necrosis in macrophages compared to the avirulent strain H37Ra, through increased disruption of the mitochondrial membrane (Chen *et al*, 2006) and manipulation of the synthesis of eicosanoids (Gan *et al*, 2008; Divangahi *et al*, 2010). Compared to H37Ra,

H37Rv also stimulates increased production of lipoxin A4 (LXA4), which promotes necrosis, but lower amounts of prostaglandin E2 (PGE2) (Chen *et al*, 2008; Urdahl *et al*, 2011). Recently, *Mtb* was also shown to prevent apoptosis in neutrophils (Blomgran *et al*, 2012). A pro-apoptotic mutant of *Mtb* induced more rapid activation of $CD4^+$ and $CD8^+$ T cells, in a neutrophil dependent manner, illustrating the importance of apoptosis in promoting the activation of the adaptive immune response (Blomgran *et al*, 2012).

1.3. Factors involved in protection against *Mtb* infection

1.3.1. $CD4^+$ T cells

In mouse models, it has been repeatedly demonstrated that $CD4^+$ T helper cells are essential for protection against *Mtb* (Flynn and Chan, 2001). Mice deficient in CD4 or MHC class II (required for the activation of $CD4^+$ T cells), and mice depleted of $CD4^+$ T cells by antibody treatment, could not control infection and had greatly reduced survival compared to control mice (Caruso *et al*, 1999; Scanga *et al*, 2000; Mogues *et al*, 2001). Arrival of $CD4^+$ T cells into the lung, at around day 16-18 post-infection, correlates with a plateau in bacteria numbers (Chackerian *et al*, 2001). $CD4^+$ are also critical for protection in humans, as killing of $CD4^+$ T cells by HIV leads to a massively increased likelihood of developing active TB (Kwan and Ernst, 2012).

1.3.2. The different subsets of effector $CD4^+$ T cells

$CD4^+$ T helper (Th) cells can be separated into subsets based on the cytokines they secrete, which define their effector functions against different classes of pathogen (Mosmann and Coffman, 1989). Effector $CD4^+$ T cells were first divided

into two subsets, Th1 and Th2 (Mosmann and Coffman, 1989). Th1 cells are responsible for the cell-mediated immune response, through the production of IFNγ, which activates macrophages in defence against intracellular pathogens (Mosmann and Coffman, 1989). Th2 cells, in contrast, produce IL-4, IL-5 and IL-13 and are involved in defence against extracellular pathogens such as helminths, through the activation of the humoral response, and the activation of mast cells and eosinophils (Sher and Coffman, 1992). More recently, a third subset has been described, Th17 cells, which produce IL-17 and act against extracellular bacteria and fungi, in part through the activation of neutrophils (Stockinger and Veldhoen, 2007).

Which subset of $CD4^+$ T cell predominates during an infection is a critical decision for the immune system, and can mean the difference between life and death for the host. This decision depends on the cytokines produced by cells of the innate immune system (O'Garra, 1998). The generation of Th1 cells requires IL-12 (Hsieh *et al*, 1993; Murphy *et al*, 2000) which triggers Th1 development through STAT4 and the transcription factor T-bet (Szabo *et al*, 2000). Th2 cells require IL-4 (Nelms *et al*, 1999) and the activation of the GATA-3 transcription factor (Zheng and Flavell, 1997; Ouyang *et al*, 1998; Ferber *et al*, 1999). Th17 cells, in contrast, require IL-6, TGF-β and IL-1 for development (Stockinger and Veldhoen, 2007; Korn *et al*, 2009), IL-23 for expansion and stabilization (Harrington *et al*, 2005) and activation of the transcription factor RORγt (Ivanov *et al*, 2006).

1.3.3. IFNγ and cell-mediated immunity is critical for protection against *Mtb*

In the case of *Mtb* infection, substantial evidence from both human and mouse studies has shown that the Th1 subset of $CD4^+$ T cells is required for

mediating protection against *Mtb* (Flynn and Chan, 2001). The evidence for this conclusion is discussed below.

1.3.3.1. IL-12 is required for control of *Mtb* infection in mice

IL-12 is a heterodimeric molecule consisting of a 35-kDa light chain (p35) and a 40-kDa heavy chain (p40), which together forms the cytokine IL-12p70 (Trinchieri, 2003). As discussed above, IL-12p70 functions to stimulate Th1 development and IFNγ production from naive $CD4^+$ T cells (Hsieh *et al*, 1993) through activation of the T-bet transcription factor (Szabo *et al*, 2000). Mice deficient in both IL-12p35 and IL-12p40 are incapable of controlling *Mtb* infection, showing that the Th1 response is critical in protection against *Mtb* (Cooper *et al*, 1997; Cooper *et al*, 2002). In support of the important role for IL-12 in IFNγ induction in T cells, mice deficient in T-bet, which is required for IL-12 stimulation of IFNγ production (Szabo *et al*, 2000), are also highly susceptible to *Mtb* infection (Sullivan *et al*, 2005).

Cooper *et al* (2002) found that IL-12p40$^{-/-}$ mice were more susceptible to *Mtb* than IL-12p35$^{-/-}$ mice, suggesting IL-12p40 itself had activities that were independent of IL-12p70. This is partially due to the fact that IL-12p40 can associate with IL-23p19 to form the cytokine IL-23 (Oppmann *et al*, 2000), as IL-23 can partially compensate for loss of IL-12p70 in stimulating naive T cells (Khader *et al*, 2005). However, IL-12p40 can also be secreted as homodimers (Trinchieri, 2003), and these have been shown to stimulate the migration of DCs from the lungs to the draining lymph nodes during *Mtb* infection, leading to the activation of the T cell response (Khader *et al*, 2006).

1.3.3.2. IFNγ activates macrophages to kill intracellular *Mtb*

Further evidence for the requirement of the Th1 response comes from studying the role of IFNγ, the hallmark cytokine produced by Th1 cells (Mosmann and Coffman, 1989). Mice deficient in IFNγ die rapidly following aerosol infection (Cooper *et al*, 1993) or intravenous infection (Flynn *et al*, 1993) with *Mtb*. The key function of IFNγ in the immune response against *Mtb* is to activate macrophages, stimulating them to kill intracellular *Mtb* (Flynn and Chan, 2001a). As discussed above, *Mtb* is able to prevent phagosome-lysosome fusion in macrophages (Vergne *et al*, 2004). However, IFNγ activates additional killing mechanisms in macrophages, and this allows the immune system to control infection (Pieters, 2008).

An important killing mechanism induced by IFNγ is the induction of the enzyme inducible nitric oxide synthase (iNOS) (Cooper, 2009). iNOS converts L-arginine into reactive nitrogen intermediates (RNIs), such as nitric oxide (NO), which have strong anti-microbial activity (MacMicking *et al*, 1997a). IFNγ can stimulate *Mtb* killing in macrophages *in vitro* through iNOS induction and RNIs (Chan *et al*, 1992), and iNOS expression *in vivo* has been shown to depend on IFNγ signalling (Flynn *et al*, 1993; Skold and Behar, 2008). Mice deficient in iNOS, either through gene knockout or treatment with iNOS inhibitors, are highly susceptible to *Mtb* infection (Chan *et al*, 1995; MacMicking *et al*, 1997b; Scanga *et al*, 2001). However, as yet no evidence for a requirement for iNOS and RNIs has been found for *Mtb* killing in human macrophages (Flynn and Chan, 2001a), although increased iNOS has been detected in alveolar macrophages taken from TB patients (Nicholson *et al*, 1996).

Other mechanisms are also activated by IFNγ that lead to *Mtb* killing in macrophages. IFNγ stimulation has been shown to overcome the block in phagosome maturation leading to lysosome-mediated *Mtb* killing (Via *et al*, 1997), which is in part dependent on the p47 GTPase family member LRG-47 (although this protein is not present in humans) (MacMicking *et al*, 2003). Mice deficient in LRG-47 were highly susceptible to *Mtb* infection, illustrating the importance of this pathway (MacMicking *et al*, 2003). More recently, induction of autophagy has been shown to mediate killing of *Mtb* in macrophages, in response to IFNγ (Gutierrez *et al*, 2004). Autophagy leads to the delivery of antimicrobial ubiquitin peptides to the lysosome, resulting in *Mtb* killing (Alonso *et al*, 2007). Finally, members of the guanylate binding protein (GBP) family of GTPases may be involved in IFNγ mediated killing. Kim *et al* (2011) found that several members of this family, including GBP1, GBP6, GBP7 and GBP10 were required for optimum IFNγ-induced killing of BCG in murine macrophages.

It has long been assumed that the major source of IFNγ during *Mtb* infection is $CD4^+$ T cells (Flynn and Chan, 2001a). However, other immune cells such as $CD8^+$ T cells, γδ T cells, NK cells and NK T cells are also capable of producing IFNγ. Recently, Gallegos *et al* (2011) showed that transfer of both WT and $IFN\gamma^{-/-}$ *Mtb*-specific $CD4^+$ T cells into WT mice led to protection against *Mtb*, suggesting that $CD4^+$ T cells may not be the major source of IFNγ. However, a recent study showed that around 70% of $IFN\gamma^+$ cells in the lung of *Mtb* infected mice were $CD4^+$ T cells, with $CD8^+$ T cells making up the remaining populations (Bold and Ernst, 2012). In addition, IFNγ production by $CD8^+$ T cells was dependent on the presence of $CD4^+$ T cells (Bold and Ernst, 2012). An alternative explanation for the results

observed by Gallegos *et al* (2011) may therefore be that $CD4^+$ T cells have additional, IFNγ-independent mechanisms for controlling *Mtb*.

1.3.3.3. Requirement for the Th1 response in human disease

Data from patients with specific genetic defects in Th1-mediated immunity has also provided strong evidence for the role of the Th1 arm in protection from mycobacterial infection. Patients with genetic defects in this arm of the immune response develop a syndrome known as Mendelian susceptibility to mycobacterial disease (MSMD) which is characterised by life-threatening infections from normally non-pathogenic mycobacteria (Casanova *et al*, 2012). Examples include disseminated BCG infection following vaccination, and infection with environmental mycobacteria such as *Mycobacterium avium* and *Mycobacterium kansasii* (Casanova *et al*, 2012).

Mutations in several genes are known to cause MSMD. These are IFNGR1 and IFNGR1, the two components of the receptor for IFNγ; STAT1, the main transcription factor that activated by IFNγ; IL12B, encoding IL-12p40; IL12RB1, a component of the IL-12 receptor; TYK2, a kinase required for several signalling pathways including IL-12; and NEMO, required for the upregulation of IL-12 via CD40 signalling (Casanova and Abel, 2002; Casanova *et al*, 2012). More recently, primary deficiency in IRF8 was found to lead to selective loss of monocytes and dendritic cells, leading to MSMD (Hambleton *et al*, 2011). Similar results have been observed in mice deficient in IRF8 (Marquis *et al*, 2011). The loss of function of all these genes leads to impaired development of, or responsiveness to, IFNγ mediated immunity, illustrating the importance of the IFNγ response in defence against mycobacteria (Casanova and Abel, 2002; Casanova *et al*, 2012).

In addition to increased susceptibility to non-pathogenic mycobacteria, deficiencies in components of Th1 immunity have, in rare cases, been linked to susceptibility to *Mtb* (Casanova and Abel, 2002). One individual with partial IFNGR1 deficiency was found to have clinical TB (Jouanguy *et al*, 1997) and severe TB has been found in several children with IL12RB1 deficiency (Altare *et al*, 2001; Caragol *et al*, 2003; Ozbek *et al*, 2005). This condition, known as Mendelian susceptibility to TB (MST), further underlines the importance of Th1 immunity in resistance against *Mtb* (Alcais *et al*, 2009).

1.3.4. The Th17 response in infection and vaccination

The cytokine IL-17 is produced by several cell types including $CD4^+$ Th17 cells, although in *Mtb* infected mice γδ T cells have been shown to be a major source (Locksley *et al*, 2001). Mice deficient in the IL-17 receptor, IL-23p19 (required for Th17 development) or treated with neutralising antibody against IL-17 had similar levels of *Mtb* in the lungs compared to control mice (Khader *et al*, 2005; Aujla *et al*, 2007; Redford *et al*, 2010). However, Redford *et al* (2010) found that anti-IL-17 treated mice had dramatically lower *Mtb* in the spleen, suggesting that IL-17 may enhance the dissemination of *Mtb* from the lung to other organs. IL-17 has also been shown to mediate pathology in *Mtb* infected mice repeatedly vaccinated with BCG (Cruz *et al*, 2010).

However, IL-17 may play a positive role in the recall response to *Mtb* following vaccination (Khader *et al*, 2007). Th17 cells appeared early in the lungs of mice vaccinated with an *Mtb* peptide, following challenge with *Mtb*, and were responsible for the subsequent recruitment of protective $IFN\gamma^+$ Th1 cells into the

lung (Khader *et al*, 2007). Th17 cells were shown to trigger the production of chemokines such as Cxcl9, Cxcl10 and Cxcl11, which stimulated the influx of IFNγ producing T cell into the lung (Khader *et al*, 2007).

1.3.5. $CD8^+$ T cells

The role of $CD8^+$ T cells in *Mtb* infection is a matter of some controversy, but it is generally accepted that they are less critical than $CD4^+$ T cell for protection (Cooper, 2009). The contribution of $CD8^+$ T cells has been addressed by infecting mice genetically deficient in several components of MHC class I pathway, which is required for the activation of $CD8^+$ T cells. The first study to address the role of $CD8^+$ T cells in *Mtb* infection used mice deficient in beta-2-microglobulin, which binds to and stabilises MHC class I molecules on the cell surface. These mice were highly susceptible to *Mtb* infection, suggesting a vital role for $CD8^+$ T cells (Flynn *et al*, 1992). However, beta-2-microglobulin also associates with the MHC class I homolog HFE, which regulates iron production, and this was shown to account for a large part of the increased susceptibility to *Mtb* (Schaible *et al*, 2002). Subsequent studies have shown, using mice specifically deficient in MHC class I (Rolph *et al*, 2001) or mice depleted of $CD8^+$ T cells by antibody treatment (Mogues *et al*, 2001), that loss of $CD8^+$ T cells leads to a relatively mild phenotype, with around a 1-log increase in *Mtb* in the lung. However, $CD8^+$ T cells may play an important role in maintaining control at later stages of infection, as depletion of $CD8^+$ T cells from mice at later timepoints led to a massive increase in *Mtb* in the lung (van Pinxteren *et al*, 2000). $CD8^+$ T cells can produce IFNγ, and can directly kill *Mtb*-infected cells,

which may explain the increased levels of bacteria observed in their absence (Serbina and Flynn, 1999; Serbina *et al*, 2000; Woodworth *et al*, 2008).

1.3.6. IL-1α and IL-1β

The IL-1 family of cytokines consists of 11 members that carry out diverse immunological functions (Sims and Smith, 2010). The best studied of these are IL-1α, IL-1β and IL-18. IL-1β is secreted primarily by monocytes, macrophages and dendritic cells, and functions to induce the production of chemokines and adhesion molecules, leading to the influx of immune cells, especially neutrophils, to the site of infection (Dinarello, 2009). IL-1β also induces cytokines, such as IL-6, acute phase proteins and vasodilation, and is important for the development of the Th17 response (Dinarello, 2009). IL-1α also possesses potent pro-inflammatory activity, however it is expressed more widely than IL-1β and is not secreted, but retained on the cell membrane (Dinarello, 2009). IL-18 is an activator of the Th1 response, synergising with IL-12 to stimulate IFNγ production from T cells and NK cells (Okamura *et al*, 1995; Ushio *et al*, 1996; Robinson *et al*, 1997). IL-1α and IL-1β both signal through the IL-1 receptor (IL-1R), and require MyD88 for their activities (Dinarello, 2009).

Given the potent pro-inflammatory effects of IL-1 family members, their production is tightly regulated, and disregulation of IL-1 production in humans can result in severe autoinflammatory disorders (Dinarello, 2009). IL-1β is regulated at two stages. First, signals through TLRs or other PRRs leads to the transcription and translation of pro-IL-1β, which is inactive and retained with the cytoplasm. The second step requires assembly of a multi-protein complex called the inflammasome which activates the enzyme caspase-1. Caspase-1 cleaves pro-IL-1β to produce

mature IL-1β, which is secreted from the cell (Schroder and Tschopp, 2010). Several proteins in the NLR family have been described to exhibit inflammasome activity, including NLRP1, NLRP3, IPAF and AIM2 (Schroder and Tschopp, 2010).

Mice deficient in the IL-1 receptor show severely increased susceptibility to *Mtb* (Juffermans *et al*, 2000; Fremond *et al*, 2007; Mayer-Barber *et al*, 2010; Mayer-Barber *et al*, 2011). IL-1α and IL-1β, despite signalling through the same receptor, play non-redundant roles in protecting against *Mtb*, as mice deficient in either cytokine show equivalent susceptibility to IL-1 receptor deficient mice (Mayer-Barber *et al*, 2011). At present, the role of IL-1 in the immune response to *Mtb* is unclear. However, mice lacking IL-1 appear to have normal levels of TNFα, IL-12 and IFNγ, suggesting a mechanism separate from effects on the cell mediated response (Cooper, 2009).

1.3.7. TNFα in mouse and man

TNFα is a cytokine produced by many cells, including macrophages and T cells, and has pleiotropic effects on the immune response, including cellular activation, cellular migration and apoptosis (Locksley *et al*, 2001). TNFα is crucial for the control of *Mtb* infection in mice; mice treated with TNFα neutralising antibodies, or mice genetically deficient in TNFα or its receptor, fail to control bacterial replication and die rapidly following infection (Flynn *et al*, 1995; Roach *et al*, 2002). TNFα is also required for the maintenance of latency, as treatment of mice with anti-TNFα antibody led to reactivation of active disease in mice (Scanga *et al*, 1999). TNFα also maintains latency in humans, as treatment of rheumatoid arthritis

and Crohn's disease patients with neutralising antibody against TNFα (Infliximab) is associated with an increased risk of TB reactivation (Keane *et al*, 2001).

However, the critical function of TNFα in the immune response to *Mtb* is unclear. *In vitro*, TNFα synergises with IFNγ to activate macrophages to kill *Mtb* (Flesch *et al*, 1994) and mice deficient in the TNFα receptor or treated with TNFα neutralising antibodies had reduced iNOS and RNI expression (Flynn *et al*, 1995). TNFα also promotes apoptosis of *Mtb* infected macrophages which, as discussed above, can limit *Mtb* spread and lead to increased T cell priming (Behar *et al*, 2011). In a zebrafish larvae model of *M. marinum* infection, TNFα was shown to restrict mycobacterial growth, in part by preventing the necrosis of *Mtb* infected macrophages (Clay *et al*, 2008). However, it has long been considered that the most important function of TNFα in the immune response to *Mtb* is to promote the formation of the granuloma (Flynn *et al*, 2011; Ramakrishnan, 2012). However, as discussed below, recent evidence has challenged this hypothesis.

1.3.8. The granuloma in *Mtb* infection

The granuloma, a compact aggregate of immune cells, is the hallmark of *Mtb* infection (Flynn *et al*, 2011). Macrophages are the major constituents of the granuloma, many of which are infected with *Mtb* (Flynn *et al*, 2011). Other immune cells such as DCs, neutrophils, T cells, B cells, NK cells and fibroblasts are also associated with granulomas (Saunders and Britton, 2007). It has long been considered that the granuloma functions to sequester *Mtb* in a compact area, in order to prevent it spreading further within the infected lung, and to other organs (Saunders and Britton, 2007). However, recent evidence, particularly from the zebrafish larvae

model of *M. marinum* infection, has suggested that the granuloma and its associated macrophages may actually promote the dissemination of *Mtb* (Ramakrishnan, 2012).

The formation of granulomas requires two key components (Saunders and Britton, 2007). First, an adaptive Th1 response is required, as shown by the fact that mice deficient in IFNγ do not generate granulomas following *Mtb* infection (Cooper *et al*, 1993). In addition, MSMD patients deficient in IFNγ or the IFNγ receptor fail to generate granulomas in infected tissues following infection with non-pathogenic mycobacteria (Casanova and Abel, 2002). Production of IFNγ by T cells is thought to mediate killing of *Mtb* within the granuloma, and thus control the infection (Saunders and Britton, 2007). The second key component in granuloma formation is the production of cytokines by cells of the innate immune system, and TNFα is thought to be the most critical. TNFα deficient mice generated poorly formed granulomas following *Mtb* infection, with high levels of necrosis and neutrophil infiltration (Bean *et al*, 1999). In addition, production of chemokines by innate immune cells is thought to lead to granuloma formation, through stimulating the recruitment of further immune cells, and TNFα has been shown to promote chemokine production (Roach *et al*, 2002). TNFα is also thought to orchestrate granuloma formation in human TB patients, and granuloma breakdown is thought to explain why patients receiving neutralising antibodies against TNFα (Infliximab) are at increased risk of reactivating latent TB (Keane *et al*, 2001).

However, the role of TNFα in granuloma formation has been challenged in recent years. Treatment of cynomolgus macaques with TNFα neutralising antibodies prior to *Mtb* infection resulted in disseminated disease, but granulomas in the lung remained intact (Lin *et al*, 2011a). In addition, zebrafish larvae deficient in TNFα

formed granulomas faster than WT controls following *M. marinum* infection, although these granulomas eventually broke down due to excessive necrosis (Clay *et al*, 2008). Thus, TNFα may be involved in maintenance, rather than formation, of granulomas.

Recent work, also using the zebrafish larvae model of *M. marinum* infection, has questioned whether the granuloma functions to prevent *Mtb* dissemination (Ramakrishnan, 2012). This was suggested by the fact that granuloma formation in this model dependent on the key mycobacterial virulence factor ESX-1 (Volkman *et al*, 2004). Subsequently, live imaging of granuloma formation in zebravish larvae has suggested that *M. marinum* induces the recruitment of macrophages to the granuloma in order to generate a fresh source of cells to infect (Davis and Ramakrishnan, 2009). These infected macrophages can then disseminate to other sites, leading to the spread of the infection (Davis and Ramakrishnan, 2009). Thus, *Mtb* and other mycobacteria may exploit the recruitment of cells to the granuloma in order to promote bacterial dissemination. However, as the zebrafish larvae model does not have an adaptive immune system, it will be important to confirm these results in models with an adaptive immune response.

1.4. Factors that regulate the immune response to *Mtb*

A potent immune response is required to efficiently clear or control an infection; however, this response must be tightly regulated to prevent an over exuberant immune response leading to immune-mediated pathology. This regulation is achieved through several specialised cells and soluble factors.

1.4.1. IL-10 and the suppression of the immune response to *Mtb*

1.4.1.1. IL-10 background

The cytokine IL-10 is an important regulator of the immune response (Moore *et al*, 2001). IL-10 signals through the IL-10 receptor (IL-10R) on target cells and mediates its activity primarily through the transcription factor STAT3 (Moore *et al*, 2001). IL-10 is produced by a wide variety of immune cells including Th1 and Th17 $CD4^+$ T cells, regulatory T cells, $CD8^+$ T cells, B cells, DCs, macrophages and neutrophils (Saraiva and O'Garra, 2010).

IL-10 was first described as a product of Th2 cells that could inhibit the production of cytokines such as IFNγ from Th1 cells (Fiorentino *et al*, 1989; Fiorentino *et al*, 1991b). This effect was subsequently shown to be indirect, via the ability of IL-10 to suppress the production of IL-12 from APCs (D'Andrea *et al*, 1993; Murphy *et al*, 1994). IL-10 can also inhibit numerous other pro-inflammatory cytokines and chemokines from macrophages and DCs, including IL-1, IL-6 and TNFα (Fiorentino *et al*, 1991a; Moore *et al*, 2001). In addition, IL-10 can inhibit antigen presentation by macrophages and DCs, through the downregulation of MHC class II and co-stimulatory molecules (de Waal Malefyt *et al*, 1991; Ding *et al*, 1993) and can inhibit macrophage killing of intracellular pathogens such as *Mtb* (Gazzinelli *et al*, 1992; O'Leary *et al*, 2011; Redford *et al*, 2011).

Although cells of the innate immune system appear to be the major targets of IL-10, recent studies have shown that IL-10 may mediate some of its suppressive activities by acting on T cells. IL-10 signalling in T regulatory cells (Tregs) was found to maintain FoxP3 expression in a mouse model of colitis (Murai *et al*, 2009). More recently, IL-10 signalling in Tregs was shown to be required for the

suppression of the Th17 response (Chaudhry *et al*, 2011). However, further work is required to determine the significance of these pathways in the immune response.

1.4.1.2. The regulation of IL-10 production in innate immune cells

IL-10 production from immune cells is regulated at multiple levels; by chromatin modifications, at the transcriptional level by a wide range of transcription factors, and post-transcriptionally through the regulation of mRNA stability (Saraiva and O'Garra, 2010). Many mechanisms of IL-10 regulation are common to several immune cells, although cell-specific mechanisms are also in place (Saraiva and O'Garra, 2010).

Many cells of the innate immune system, including macrophages, DCs and neutrophils, produce IL-10 in response to pathogens or pathogen derived products, through triggering of PRRs (Boonstra *et al*, 2006; Zhang *et al*, 2009). Stimulation of macrophages and DCs with TLR3, TLR4 and TLR9 agonists leads to IL-10 production from macrophages and myeloid DCs, and involved both the MyD88 and TRIF dependent pathways (Boonstra *et al*, 2006). In addition to TLRs, the C-type lectins dectin-1 (LeibundGut-Landmann *et al*, 2007) and DC-SIGN (Geijtenbeek and van Kooyk, 2003; Gringhuis *et al*, 2007) can stimulate IL-10 production in innate immune cells.

A key pathway for IL-10 downstream of PRR signalling is the extracellular signal-related kinase (ERK) MAP kinase pathway (Saraiva and O'Garra, 2010). Activation of ERK in response to TLR signalling requires the upstream MAP kinase family members tumour progression locus 2 (TPL2) and MEK1/2 (Symons *et al*, 2006) and macrophages deficient in *Tpl2*, or treated with MEK1/2 inhibitors, produce markedly less IL-10 compared to controls in response to TLR ligands (Yi *et*

al, 2002; Agrawal *et al*, 2003; Dillon *et al*, 2004; Kaiser *et al*, 2009). The strength of ERK activation appears to correlate with IL-10 production in macrophages, myeloid DCs and pDCs (Kaiser *et al*, 2009). Activation of ERK is highest in macrophages following TLR stimulation, with moderate levels in myeloid DCs and undetectable amounts in pDCs (Kaiser *et al*, 2009). Downstream of ERK signalling, the transcription factor c-Fos can mediate IL-10 induction (Agrawal *et al*, 2003; Dillon *et al*, 2004; Kaiser *et al*, 2009). ERK is also required for induction of IL-10 downstream of dectin-1 in DCs and neutrophils, through a pathway involving Syk and Card9 (Slack *et al*, 2007; Zhang *et al*, 2009; Dorhoi *et al*, 2010). ERK signalling has also been shown to promote IL-10 production from several $CD4^+$ T cell subsets (Saraiva *et al*, 2009).

Many other pathways have also been implicated in IL-10 production in innate immune cells (Saraiva and O'Garra, 2010). Downstream of LPS stimulation in macrophages, NF-κB p65 was recruited to a DNase1 hypersensitivity site in the IL-10 promoter, and this was required for optimal IL-10 production (Saraiva *et al*, 2005). NF-κB p50 homodimers are also recruited to the proximal IL-10 promoter, and macrophages from p50 deficient mice show impaired IL-10 production in response to LPS (Cao *et al*, 2006). In addition, and in keeping with a requirement for the TRIF-dependent pathway (Boonstra *et al*, 2006), type I IFN has been shown to promote IL-10 production in macrophages, in response to LPS (Chang *et al*, 2007; Iyer *et al*, 2010).

1.4.1.3. The role of IL-10 in infectious disease

Given the potent anti-inflammatory properties of IL-10, the impact of IL-10 in the immune response to pathogens has been the focus of much study, primarily

using mice genetically deficient in IL-10 (*Il10*$^{-/-}$ mice). Several studies have shown that IL-10 can play a detrimental role in the immune response to infection. Dai *et al* (1997) found that *Il10*$^{-/-}$ mice had greatly reduced bacterial loads following infection with *Listeria monocytogenes*, and this was associated with a greatly increased Th1 response and higher levels of IL-1, TNFα and IL-12. Similar results have been reported in mice infected with *Leishmania major* (Belkaid *et al*, 2001). Whereas WT mice failed to clear the parasites, leading to a chronic infection, *Il10*$^{-/-}$ mice, and mice treated with IL-10R blocking antibodies, were able to clear the parasites, showing that the activities of IL-10 can result in pathogen persistence (Belkaid *et al*, 2001).

However, in other situations IL-10 is vital in protecting the host from immune-mediated pathology. Although *Il10*$^{-/-}$ mice infected with *Plasmodium chabaudi* produced increased levels of IFNγ, TNFα and IL-12, this did not lead to improved pathogen clearance (Li *et al*, 1999). Instead, *Il10*$^{-/-}$ mice developed a much more severe infection compared to WT controls, with increased fever and weight-loss, and significant mortality (Li *et al*, 1999). Similar results were reported in the context of *Toxoplasma gondii* infection, with *Il10*$^{-/-}$ mice developing severe immune-mediated pathology and suffering severe mortality compared to WT controls, despite a greatly enhanced Th1 response (Gazzinelli *et al*, 1996).

1.4.1.4. The role of IL-10 in *Mtb* infection

IL-10 can inhibit several aspects of the immune response which are critical for protection against *Mtb* infection (Moore *et al*, 2001; Redford *et al*, 2010). IL-10 can suppress the production of IL-12 from macrophages and DCs, leading to suppression of IFNγ production from T cells (Fiorentino *et al*, 1991b; D'Andrea *et*

al, 1993; Moore *et al*, 2001), a process which is crucial in protection against *Mtb* (Flynn and Chan, 2001a). In addition, IL-10 suppresses IL-1 and TNFα production from innate immune cells (Fiorentino *et al*, 1991a), and both these cytokines are needed to control *Mtb* infection (Flynn *et al*, 1995; Mayer-Barber *et al*, 2011). A large number of studies have addressed the effect of IL-10 on the response to *Mtb*, in both mice and human infection, but the results have often been contradictory (Redford *et al*, 2011). A summary of the effects of IL-10 on the immune response to *Mtb* is shown in Figure 1.4.

1.4.1.4.1. Results from the mouse model

A number of studies have addressed the role of IL-10 in *Mtb* infection by infecting *Il10*$^{-/-}$ mice with *Mtb*, and comparing the subsequent immune response and bacterial loads to WT mice. Some studies have reported no difference in bacterial burden in the lungs between WT and *Il10*$^{-/-}$ mice following *Mtb* infection (North, 1998; Jung *et al*, 2003) suggesting that IL-10 may not play an important role in infection. A subsequent study found that although IL-10 did not affect *Mtb*-bacterial load, it protected mice against fatal immune-pathology, although this only occurred at late stages of infection (Higgins *et al*, 2009).

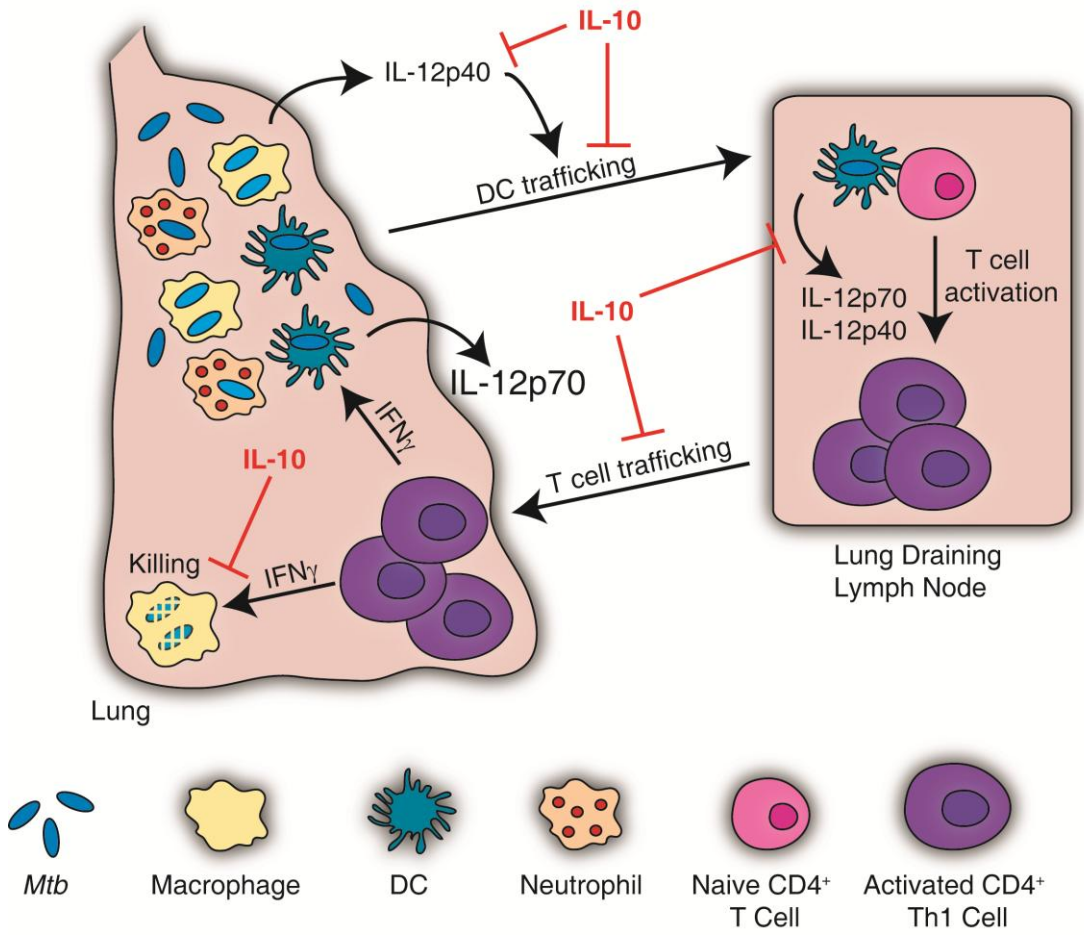

Figure 1.4. The effects of IL-10 on the immune response to *Mtb*

However, other studies have found decreased burdens of *Mtb* in *Il10*$^{-/-}$ mice (Roach *et al*, 2001; Beamer *et al*, 2008; Redford *et al*, 2010). In these studies, the increased resistance to *Mtb* in *Il10*$^{-/-}$ mice correlated with an enhanced Th1 response, including increased production of IFNγ (Roach *et al*, 2001; Beamer *et al*, 2008; Redford *et al*, 2010). Levels of TNFα, IL-17, GM-CSF and IP-10 were also increased in the serum of *Il10*$^{-/-}$ mice infected with *Mtb* (Redford *et al*, 2010). A further study used transgenic mice which over-produced IL-10 under the control of the IL-2 promoter, and found that this increased IL-10 production led to failure to control *Mtb* at later stages of infection (Turner *et al*, 2002).

These differences observed between these studies are likely to be due to variation in the strain of mouse used, strain of *Mtb* used and route of infection (Redford *et al*, 2011). In addition, the majority of these studies were carried out using C57Bl/6 and BALB/c mice, which are relatively resistant to *Mtb* infection, and may therefore not provide the best model for human TB (Redford *et al*, 2011). The CBA/J mouse is dramatically more susceptible to *Mtb*, and produce increased levels of IL-10 following infection (Turner *et al*, 2002). Importantly, Redford *et al* (2010) showed that blockade of IL-10 signalling during *Mtb* infection in CBA/J mice can reduce *Mtb* levels in the lungs, and enhance the Th1 response. IL-10 may therefore play an important role in more susceptible mouse models of *Mtb*.

1.4.1.4.2. IL-10 in human TB

Several studies have found that IL-10 mRNA and protein are detectable in the lungs of patients with active TB (Barnes *et al*, 1993; Bonecini-Almeida *et al*, 2004; Almeida *et al*, 2009). In addition, neutralisation of IL-10 during stimulation of PBMCs from PPD^+ positive donors led to enhanced T cell proliferation and IFNγ production, suggesting that IL-10 can suppress *Mtb* specific T cell responses during human TB (Gong *et al*, 1996; Rojas *et al*, 1999). However, although a large number of studies have been carried out addressing whether IL-10 polymorphisms are associated with reduced or increased protection against *Mtb*, results have generally been inconclusive (Redford *et al*, 2011).

1.4.2. Suppression of the immune response to *Mtb* by Tregs

Regulatory T cells (Tregs) are another important mechanism for the regulation of the immune response. These cells are important for the prevention of

autoimmunity, and can also regulate the immune response to infection, in order to prevent immunopathology (Fehervari and Sakaguchi, 2004; Belkaid and Tarbell, 2009). Tregs are induced during the immune response to *Mtb* in both mice and humans, and can regulate the induction of the protective immune response (Urdahl *et al*, 2011). The transfer of *Mtb*-specific Tregs into *Mtb* infected mice at day 11 post-infection resulted in increased bacterial growth at later timepoints, and delayed the expansion of effector T cells (Shafiani *et al*, 2010). In addition, co-transfer of Tregs with $CD4^+$ T cells into $RAG^{-/-}$ infected with *Mtb* blocked the protective effects of $CD4^+$ T cells (Kursar *et al*, 2007). Furthermore, depletion of Tregs from mice infected with *Mtb* results in lower bacterial loads in the lung and spleen, and was accompanied by increased IFNγ production by $CD4^+$ T cells (Scott-Browne *et al*, 2007). Thus, Tregs appear to play an important regulatory role during *Mtb* infection by specifically targeting the T cell response (Urdahl *et al*, 2011).

1.5. Tuberculosis and type I IFN

1.5.1. Background

There are three families of IFN, known as type I, II and III. IFNγ is the only member of the type II IFN family, and is known to be a crucial component in the immune response to *Mtb* due to its stimulatory effects on macrophages (Flynn and Chan, 2001a). The type I IFNs consist of more than 20 IFNα genes and a single IFNβ gene. There are several further subtypes of type I IFNs, such as IFNκ, IFNδ, IFNε and IFNτ, but these are only expressed in specific tissues and are not found in all species (Theofilopoulos *et al*, 2005). The more recently discovered type III IFNs, also known as IFNλ, consist of IFNλ1, IFNλ2 and IFNλ3 (also known as IL-29, IL-

28A and IL-28B) in humans, and IFNλ2 and IFNλ3 in mice (Donnelly and Kotenko, 2010).

The type I IFNs were discovered in 1957 as soluble factors capable of inhibiting viral replication (Isaacs and Lindenmann, 1957). Almost all cells of the body are capable of producing and responding to type I IFN, although some, such as pDCs, are specialised to secrete large amounts (Asselin-Paturel *et al*, 2001; Colonna *et al*, 2004). Although long regarded as purely antiviral effectors, the type I IFN are now known to regulate a wide range of immune functions, and play a role in a number of bacterial infections, including *Mtb* (Trinchieri, 2010).

1.5.2. The Regulation of type I IFN production

The master regulators of type I IFN production are the transcription factors IRF3 and IRF7 (Decker *et al*, 2005). IRF3 is expressed constitutively, and serine phosphorylation of IRF3, either by TBK-1 or IKKε, causes it to dimerise and migrate to the nucleus where, along with other transcription factors, it activates transcription of the IFNβ gene (Decker *et al*, 2005). Signalling by low levels of IFNβ leads to activation of IRF7, which then stimulates the expression of IFNα and the further expression of IFNβ (Marie *et al*, 1998; Sato *et al*, 1998).

Various bacterial and viral components trigger type I IFN production in immune cells. TLR3 and TLR4, in response to double-stranded viral RNA and bacterial LPS respectively, activate IRF3 via the TRIF dependent pathway (Kawai and Akira, 2010). Double-stranded viral RNA also activates the cytoplasmic receptors RIG-I and MDA-5, leading to IRF3 activation through MAVS and TBK1 (Kawai and Akira, 2010). Finally the NLRs, NOD1 and NOD2, trigger type I IFN in

response to components of peptidoglycan (Trinchieri, 2010) and it is this NLR-dependent pathway which is thought to mediate type I IFN induction in response to *Mtb*, through Rip2 and IRF5 (Pandey *et al*, 2009).

However, type I IFN induction in pDCs appears independent of the amplification loop that operates in other cells. Instead, single-stranded RNA and CpG stimulate the rapid activation of IRF7 through TLR7 and TLR9, and induce high levels of IFNα and IFNβ through a MyD88-dependent pathway (Colonna *et al*, 2004; Guiducci *et al*, 2006). This difference is due to the trafficking of CpG to different endosomes in pDCs compared to conventional DCs (Honda *et al*, 2005). However, the type of CpG used to stimulate pDCs affects whether high levels of IFNα are induced; CpG-A localises to early endosomes and induces high levels of IFNα, whereas CpG-B localises to late endosomes and does not induce IFNα (Guiducci *et al*, 2006).

1.5.3. IFN signalling

The three families of IFNs signal through distinct receptors, but share many downstream signalling molecules and induce overlapping gene expression (Der *et al*, 1998; Stark *et al*, 1998; Donnelly and Kotenko, 2010). Signalling by the IFNs is summarised in Figure 1.5. All members of the type I IFN family signal through a common receptor, a heterodimeric molecule consisting of IFNAR1 and IFNAR2 (known as the IFNαβR), and the downstream signalling from this receptor has been extensively characterised (Stark *et al*, 1998) (Figure 1.5). In response to binding, the two subunits of the IFNαβR change conformation and dimerise, resulting in the phosphorylation and activation of the receptor associated Janus family kinases Tyk2

and Jak1, and the phosphorylation of the intracellular domain of the IFNαβR (Stark *et al*, 1998; Decker *et al*, 2005). This creates a docking site for STAT1 and STAT2 molecules, which are then phosphorylated by Jak1 and Tyk2, causing them to form STAT1:STAT2 heterodimers and migrate to the nucleus, where they associate with the transcription factor IRF9 to form the interferon-stimulated gene factor 3 (ISGF3) complex (Stark *et al*, 1998; Decker *et al*, 2005). The ISGF3 binds to a consensus sequence, the interferon stimulated response element (IRSE), in the promoters of interferon stimulated genes (ISGs) to activate their transcription (Stark *et al*, 1998; Decker *et al*, 2005).

IFNγ signals through a receptor consisting of IFNGR1 and IFNGR1. Ligand binding results in activation of Jak1 and Jak2, and the predominant transcriptional complex activated by IFNγ are homodimers of STAT1. This complex migrates to the nucleus and activates the transcription of genes with an IFNγ-activated site (GAS) in their promoter (Stark *et al*, 1998). Type I IFN can also stimulate the formation of STAT1 homodimers, leading to overlap between IFNγ and type I IFN inducible genes (Platanias, 2005) (Figure 1.5).

The three subtypes of IFNλ signal through a separate receptor to type I IFN, consisting of a unique IFNλ-R1 receptor subunit coupled to IL-10R2 (Donnelly and Kotenko, 2010). However, the IFNλ receptor, like the IFNαβR, is associated with Jak1 and Tyk2, and also activates the ISGF3 complex, inducing very similar gene expression to type I IFN, and similar antiviral activity (Donnelly and Kotenko, 2010).

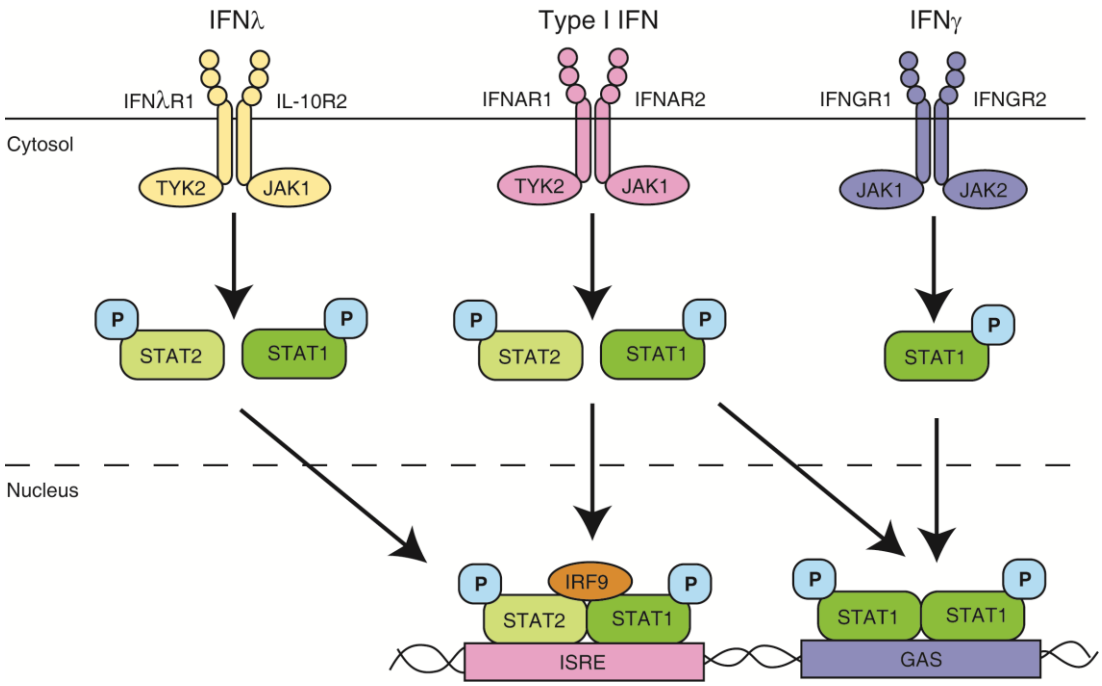

Figure 1.5. An overview of signalling pathways activated by IFNs. Adapted from Platanias *et al*, (2005)

1.5.4. Type I IFN in protection against viral infection

Type I IFNs were discovered, and named, on the basis of their ability to interfere with viral replication (Isaacs and Lindenmann, 1957) and mice deficient in type I IFN through the deletion of the IFNAR1 subunit of the IFNαβR (*Ifnar1*$^{-/-}$ mice) are highly susceptible to certain viral infections (van den Broek *et al*, 1995; Theofilopoulos *et al*, 2005).

This activity is due to the potent antiviral genes induced by type I IFN. Prominent among these are protein kinase R (PKR), 2'5'oligoadenlyate synthetase (2'5'OAS), RNAse L and Mx proteins (Sadler and Williams, 2008). These proteins inhibit viral replication in a variety of ways, and mice deficient in these genes are susceptible to viral infection (Sadler and Williams, 2008). Protein kinase R is a serine threonine kinase that phosphorylates the translational initiation factor eiF2,

leading to inhibition of translation (Stark *et al*, 1998; Sadler and Williams, 2008). 2'5'OAS enzymes produce short 2'5'oligoadenylate molecules which activates the RNAse L, which in turn destroys viral dsRNA. Type I IFNs also induce many members of the TRIM family of proteins (Rajsbaum *et al*, 2008), some of which have antiviral activity (McNab *et al*, 2010). However, the understanding of the anti-viral effects of type I IFNs is still in its infancy, as the functions of the vast majority of type I IFN inducible genes are not known (Stetson and Medzhitov, 2006).

1.5.5. Type I IFN in bacterial infection

In recent years, the recognition that type I IFN can affect many immune processes besides mediating antiviral immunity has led to investigations into the role of type I IFN in bacterial infection (Trinchieri, 2010). Broader functions of type I IFNs include activating DCs in response to TLR ligands (Asselin-Paturel *et al*, 2005), stimulating DCs to cross-present antigen to $CD8^+$ T cells (Le Bon *et al*, 2003), enhancing the survival of activated T cells (Marrack *et al*, 1999), inducing IFNγ in $CD8^+$ T cells (Cousens *et al*, 1999) and NK cells (Hunter *et al*, 1997), and regulating the humoral immune response (Le Bon *et al*, 2001).

Studies investigating the role of type I IFN in bacterial infection have made use of mice lacking the receptor for type I IFN (*Ifnar1*$^{-/-}$ mice). These mice have been challenged with a variety of bacterial pathogens, and it has become clear that type I IFN can have both positive and negative effects for the host, depending on the infection (Bogdan *et al*, 2004; Decker *et al*, 2005; Trinchieri, 2010). *Ifnar1*$^{-/-}$ mice challenged with group B streptococcus (GBS), *Streptococcus pneumoniae* and *Escherichia coli* all had increased mortality compared to wild-type controls, showing

an essential role for type I IFNs in response to these pathogens (Mancuso *et al*, 2007). Type I IFN was also found to be essential for defence against *Streptococcus pyogenes* (Gratz *et al*, 2011).

However, in some infections type I IFN can play a negative role in host resistance to infection. This first became apparent in the context of *Listeria monocytogenes* infection. Three papers published in 2004 showed that *Ifnar1*$^{-/-}$ mice infected with *Listeria* had reduced bacterial burdens in the spleen and liver compared to wild-type mice, showing that in the absence of type I IFN, the immune response is more effective (Auerbuch *et al*, 2004; Carrero *et al*, 2004; O'Connell *et al*, 2004). The mechanism behind these negative effects of type I IFN is unclear. Two of these studies found that apoptosis of splenic lymphocytes was reduced in *Ifnar1*$^{-/-}$ mice (Carrero *et al*, 2004; O'Connell *et al*, 2004). In addition, Auerbuch *et al* (2004) showed that the levels of IL-12p70 in the serum of *Ifnar1*$^{-/-}$ mice are elevated compared to wild-type mice, suggesting that type I IFNs negatively regulate IL-12. More recently, Rayamajhi *et al* (2010) showed that type I IFN could downregulate the expression of the IFNγ receptor (specifically IFNGR1) on the surface of *Listeria monocytogenes* infected macrophages, leading to a reduced responsiveness to IFNγ in these cells.

Similar results have recently been demonstrated in the context of *Francisella tularensis* infection, with *Ifnar1*$^{-/-}$ mice showing enhanced bacterial clearance (Henry *et al*, 2010). In this study, type I IFN was suggested to suppress IL-17 production from γδ T cells (Henry *et al*, 2010). In general, type I IFN seems to play a protective role against extracellular bacteria, such as *Streptococci*, but a negative role against intracellular bacteria, such as *Listeria monocytogenes* and *Francisella tularensis*.

1.5.6. Type I IFN in *Mtb* infection

The role of type I IFN in *Mtb* infection has been the subject of much investigation, mostly using mouse models of *Mtb* infection. However, contradictory results have been observed, with some studies reporting a beneficial role for type I IFN in the immune response, whilst others have found a detrimental role. The results of these studies are summarised in Table 1.1 at the end of this section. As shown in Table 1.1, the majority of studies suggest that type I IFN hinders the immune response to *Mtb*.

A study by Manca *et al* (2001) compared infection of mice with HN878, a hypervirulent strain of *Mtb*, with other less virulent strains. They found that HN878 induced higher levels of type I IFN from mouse lungs, but a less potent Th1 response (Manca *et al*, 2001). Treatment of mice with recombinant type I IFN led to reduced survival times following *Mtb* infection (Manca *et al*, 2001). The authors concluded that virulent strains of *Mtb* induce type I IFNs as a pathogenic mechanism, in order to blunt the crucial Th1 response (Manca *et al*, 2001). A further study by the same group showed that another hypervirulent strain, W4, also induced more type I IFN in the lungs of mice compared to non-hypervirulent strains (Manca *et al*, 2005). This study also found that *Ifnar1*$^{-/-}$ mice survived longer than wild type mice following *Mtb* infection, showing that mice are better protected against *Mtb* in the absence of type I IFN signalling (Manca *et al*, 2005). Subsequently, Ordway *et al* (2007) found that *Ifnar1*$^{-/-}$ mice infected with five different *Mtb* strains showed decreased bacterial counts in the lung at later time-points post-infection. However, there was no difference in the survival of these mice (Ordway *et al*, 2007). Stanley *et al* (2007) also compared resistance of wild-type and *Ifnar1*$^{-/-}$ mice to *Mtb* infection and found

that *Ifnar1*$^{-/-}$ mice had reduced bacterial burdens in the spleen, but the lung was not affected. In general, all of these studies reported decreased bacterial burdens in the lungs or spleen of *Ifnar1*$^{-/-}$ mice compared to wild-type, suggesting that type I IFN plays a negative role during *Mtb* infection. In support of this conclusion, mice deficient in a prominent ISG, protein kinase R (PKR), were also shown to have reduced burdens of *Mtb* following infection (Wu *et al*, 2012). PKR was shown to promote IL-10 production from macrophages infected with *Mtb*, and inhibit responsiveness to IFNγ (Wu *et al*, 2012).

More recently, a novel strategy was used to study the effects of type I IFN in *Mtb* infection. Antonelli *et al* (2010) treated *Mtb*-infected mice intranasally with poly-IC, an analogue of dsRNA that can induce large amounts of type I IFN from immune cells through TLR3. They found that mice treated with Poly-IC had a dramatic increase in bacterial load in the lung, accompanied by increased lung pathology and decreased survival (Antonelli *et al*, 2010). Importantly, this effect was not seen in *Ifnar1*$^{-/-}$ mice, demonstrating that the negative effects of Poly-IC were type I IFN dependent (Antonelli *et al*, 2010). Treatment of mice with Poly-IC was accompanied by the CCR2-dependent migration of a myeloid population to the lung, which seemed to be permissive for *Mtb* growth (Antonelli *et al*, 2010).

Limited evidence correlates type I IFN with beneficial effects in *Mtb* infection (Table 1.1). Some studies with *Ifnar1*$^{-/-}$ mice have shown reduced resistance to infection with mycobacteria, although they conflict with other studies described above. Kutchey *et al* (2006) showed that *Ifnar1*$^{-/-}$ mice had increased growth of BCG early in infection and Cooper *et al* (2000) found similar results with *Mtb* Erdman, suggesting that type I IFN may be required for an optimum immune

response to *Mtb*. The differences between these and other studies may be a result of the use of differing strains of mice, strains of mycobacteria and routes of infection. More recently, Desvignes *et al* (2012) generated mice deficient in both type I IFN and IFNγ signalling, and found that these mice showed greater susceptibility to *Mtb* compared to IFNγ single knockout mice. The authors concluded that type I IFN can play a protective role in the absence of IFNγ signalling, possibly through partial compensation of IFNγ activities (Desvignes *et al*, 2012).

Recently, the potential role of type I IFN in human disease was highlighted. Berry *et al* (2010) carried out whole-blood transcriptional profiling of patients with active and latent TB, as well as healthy controls, and found a 393 gene signature in the blood of active patients, which correlated with disease severity and was diminished upon antibiotic treatment. Modular and pathway analysis of this signature showed it to contain genes associated with both type I IFN and IFNγ signalling. By carrying out analysis of separated cells, this signature was found to be specific to neutrophils and macrophages (Berry *et al*, 2010). A subsequent independent study also found an upregulation of IFN inducible genes in the blood of active TB patients (Maertzdorf *et al*, 2011). However, the effects of type I IFN signalling in human TB patients are unclear.

Reference	Experimental strategy	Mouse Strain	Route of *Mtb* infection	Strain of *Mtb*	Read-out	Concluded role of type I IFN
Cooper *et al* 2000	WT vs. *Ifnar1*$^{-/-}$ mice	B6/129	Aerosol	Erdman	Lung CFU	Positive – early increase in *Mtb* levels in *Ifnar1*$^{-/-}$ mice
Manca *et al* 2001	Intranasal treatment with recombinant IFNαβ	B6D2/F_1	Aerosol	HN878 and CDC1551	Survival and lung CFU	Negative – reduced survival time in IFNαβ treated mice, and increased lung CFU
Manca *et al* 2005	WT vs. *Ifnar1*$^{-/-}$ mice	B6D2/F_1	Aerosol	HN878	Survival and Lung CFU	Negative – longer survival in *Ifnar1*$^{-/-}$ mice
Kutchey *et al* 2006	WT vs. *Ifnar1*$^{-/-}$ mice	129S6/SvEv	Aerosol	BCG	Lung CFU	Positive – early increase in BCG levels in *Ifnar1*$^{-/-}$ mice
Ordway *et al* 2007	WT vs. *Ifnar1*$^{-/-}$ mice	C57Bl/6 controls, A129 *Ifnar1*$^{-/-}$	Aerosol	H37Rv, HN878, CSU123. CSU93 Erd KO1	Survival and Lung CFU	Negative – increased lung CFU in *Ifnar1*$^{-/-}$ mice but no change in survival
Stanley *et al*, 2007	WT vs. *Ifnar1*$^{-/-}$ mice	C57Bl/6	Intravenous	Erdman	Lung/Spleen CFU	Negative – increased CFU in the spleen
Antonelli *et al*, 2010	Intranasal treatment with Poly-ICLC	C57Bl/6	Aerosol	H37Rv	Survival and Lung CFU	Negative – reduced survival time and increased *Mtb* in Poly-ICLC treated mice
Desvignes *et al*, 2012	IFNγR$^{-/-}$ vs. *Ifnar1*$^{-/-}$ IFNγR$^{-/-}$ mice	C57Bl/6	Aerosol	H37Rv	Survival and Lung CFU	Positive – could play a protective role in the absence of IFNγ

Table 1.1. Summary of previous literature investigating the role of type I IFN in *Mtb* infection

1.6. Microarray as a tool for studying host-pathogen interactions

1.6.1. Background

A common aim in biological research is to determine which genes change expression under different biological conditions. Well-studied examples include studying the responses of cells to a given stimulus over time or comparing healthy and diseased tissues. Knowledge of the genes that change under these differing conditions can give insights into the mechanisms behind these processes. For example, if a gene is upregulated in cancerous tissue compared to healthy tissue, this may indicate oncogenic properties of this gene. Techniques for studying gene expression involve detecting and quantifying the amounts of a given mRNA in a sample, using techniques such as PCR. However, these strategies, although powerful, are limited by the fact that the expression of only a small number of genes could be determined. Furthermore, this results in bias, where experimenters determine the expression of genes they already predict may be involved. The advent of DNA microarray technology in the mid-1990s revolutionised the study of gene expression, as it allowed the simultaneous and rapid quantification of thousands of mRNA species from a biological sample, and thus provided a global and unbiased picture of gene expression. Although the generation of such large amounts of data has its own problems and obstacles, particularly in regard to data analysis and statistical methods, the large number of published microarray studies revealing insights into various biological processes is testament to the power of this technique (Jenner and Young, 2005; Pascual *et al*, 2010).

1.6.2. Analysis of gene expression by microarray

A DNA microarray consists of a solid surface known as a chip, typically made of glass or plastic, onto which short single-stranded DNA sequences are added. These DNA sequences are known as probes, and each binds to a complementary mRNA sequence. Analysis of gene expression by microarray includes several stages. First, total mRNA must be extracted and purified from the biological samples in question. This mRNA is then amplified, to give a sufficient amount of material for analysis, and then labelled with a specific fluorescent marker to allow detection. The labelled mRNA is then hybridised with the microarray chip in order to allow binding of the DNA probes with complementary mRNA species. Thus, if the complementary mRNA of a given DNA probe is present in a sample, it will bind to the complementary probe, and binding can be detected by exciting the fluorescent tags on the mRNA molecules with light of the appropriate wavelength. The chip is then scanned to generate an image showing the points of fluorescence on the chip and the image is processed to numerically determine the relative intensity at each of the probes on the chip (Heller, 2002).

There are a number of different microarray platforms available from different companies (Heller, 2002). The differences between the platforms involve how the DNA probes are attached to the microarray surface. This is achieved either through deposition, in which the DNA probes are synthesised separately and then deposited onto the surface, or *in situ* synthesis, where the probes are synthesised directly onto the chip surface (Heller, 2002).

1.6.3. Illumina BeadArray technology

A recently developed and widely used technology for depositing DNA probes onto a microarray chip is BeadArray technology developed by Illumina (Illumina, 2005), unlike previous technologies such as Affymetrix technology, which attached the probes directly to the chip by *in situ* synthesis, BeadArray technology attaches multiple copies of each 50 base-pair oligonucleotide probe to silica beads of approximately 3μm in diameter (microbeads) (Illumina, 2005). These beads are then randomly self-assembled into microwells on the surface of the chip, in a defined grid pattern, with each bead located approximately 5.7μm from adjacent beads (Illumina, 2005). Importantly, each probe contains both a region for mRNA binding and an "address" sequence. The address sequence allows the location of each bead, and therefore each probe, on the chip to be determined, a process known as decoding (Illumina, 2005). This information is then used to determine which probes have bound their complementary mRNA, following hybridisation of the chips with biotin-labelled mRNA. Additionally, approximately 30 identical beads with the same probe are present on each chip, meaning that the abundance of each mRNA species is measured multiple times, increasing the accuracy of the measurement (Illumina, 2005).

1.6.4. The application of microarray in studying the immune response

Since its development, DNA microarray technology has been used to study a wide range of diseases and conditions. Approaches have included the analysis of samples from patients with various conditions, as well as more specific interactions using *in vitro* assays. The power of microarray in these scenarios is the ability to

generate a global analysis of transcription, which can lead to the generation of new hypotheses and candidate genes or pathways that were previously unsuspected to be involved.

The use of microarray in studying disease was first proposed in a study by Golub *et al* (1999), where investigators found gene signatures that could discriminate between patients with acute myeloid leukaemia and acute lymphoblastic leukaemia. These techniques have subsequently been applied to a range of different cancers. For example, transcriptional profiling is now used to distinguish different classes of breast cancer, and to predict patient outcome and response to chemotherapy (Sotiriou and Pusztai, 2009). Microarray has also been applied to the study of autoimmune diseases, and has yielded new insights into the pathogenesis of previously poorly understood conditions (Pascual *et al*, 2010). Transcriptional profiling of peripheral blood mononuclear cells (PBMCs) from patients with the autoimmune condition systemic lupus erythematosus (SLE) revealed a signature associated with type I IFN signalling and neutrophils (Bennett *et al*, 2003). Type I IFN was subsequently shown to prime neutrophils to undergo netosis, resulting in the release of neutrophil extracellular traps (NETs), resulting in the generation of the auto-antibodies against nuclear antigens, which lead to SLE pathogenesis (Garcia-Romo *et al*, 2011). Similar approaches were applied to systemic onset juvenile idiopathic arthritis (SoJIA), an autoimmune condition affecting children and characterised by fever, rash and arthritis (Pascual *et al*, 2010). Transcriptional profiling of PBMCs from SoJIA patients revealed an upregulation of IL-1β production, and treatment of patients with anakinra, an IL-1 receptor antagonist, dramatically improves the symptoms of SoJIA patients (Pascual *et al*, 2005).

These techniques have also been applied to infectious diseases, leading to novel insights into disease pathogenesis. Blood signatures were shown to discriminate patients with influenza, *E. coli* and *S. aureus* infection, which may aid in the differential diagnosis of these conditions (Ramilo *et al*, 2007). As discussed above, profiling of the whole blood of patients with active and latent TB compared to healthy controls found an IFN signature specific to patients with active TB (Berry *et al*, 2010). This could potentially aid in the diagnosis of active TB, which is not straightforward (Barry *et al*, 2009), and also reveals a potential role for type I IFN in disease pathogenesis (Berry *et al*, 2010). These studies demonstrate that microarray can be effectively applied to generate new insights into disease pathogenesis, and to potentially diagnose disease and predict disease outcome.

However, microarray has also been extensively used to study specific disease processes at the cellular level. This has been effective in answering more specific questions about the behaviour of certain cells and the functions of signalling pathways. Many studies have applied this approach to understand the interactions between immune cells and pathogens or pathogen-derived products, and this has shed light on how immune cells respond to pathogens, the signalling pathways involved in these responses, and how pathogens attempt to subvert these processes (Jenner and Young, 2005).

Microarray was first used to study host-pathogen interactions by Zhu *et al* (1998), using human fibroblasts infected with human cytomegalovirus. Since then many studies have profiled the response of a diverse array of cell types to various pathogens, including viruses, bacteria, protozoa and fungi (Jenner and Young, 2005). A common theme to emerge from these studies is a large amount of overlap in the transcriptional response of immune cells to different pathogens (Jenner and Young,

2005). For example, Nau *et al* (2002) stimulated human macrophages with E.*coli*, S. *typhi*, S. *typhimurium*, S. *aureus*, L. *monocytogenes*, *Mtb* and BCG, and identified a common response including cytokines, chemokines, transcription factors and cell-surface receptors. A similar "core response" was found by a large-scale meta-analysis of published host-pathogen microarray studies (Jenner and Young, 2005). The reasons for this overlap reflects the fact that diverse pathogens can all activate TLR signalling pathways, which culminate in the activation of common transcription factors (Jenner and Young, 2005). This is reflected in the fact that purified TLR ligands such as LPS can recapitulate a large part of the macrophage activation programme (Nau *et al*, 2002). However, pathogen-specific responses have also been identified by microarray studies of infected immune cells. For example, Nau *et al* (2002) found that *Mtb* induced lower levels of mRNA for IL-12p40 and IL-15 compared to other pathogens. In addition, Chaussabel *et al* (2003) found pathogen specific response to a variety of intracellular pathogens, including *Mtb*, in human macrophages and DCs.

A further application of microarray is to study the role of specific molecules and signalling pathways by making use of mouse gene knockout technology (Jenner and Young, 2005). In these experiments, the response of immune cells from WT mice can be compared to cells from mice deficient in particular molecules, and thus the genes regulated by a particular molecule can be determined globally (Jenner and Young, 2005). For example, Gilchrist *et al* (2006) used microarray and other systems biology techniques to identify the transcription factors ATF3 as a negative regulator of the macrophage response to LPS. This was confirmed by stimulating *Atf3*$^{-/-}$ macrophages with LPS and analysing the transcriptional response by microarray,

which found that a number of pro-inflammatory mediators such as IL-12, TNF and iNOS were upregulated in *Atf3*$^{-/-}$ macrophages (Gilchrist *et al*, 2006).

A number of microarray studies have investigated the transcriptional response of innate immune cells to *Mtb* infection. A recent study found that type I IFN inducible genes were upregulated in human macrophages infected with *Mtb* (Wu *et al*, 2012), in keeping with the IFN signature observed in active TB patients (Berry *et al*, 2010). Microarray analysis of *Mtb*-infected murine macrophages found that genes involved in steroid biosynthesis were upregulated in response to the hypervirulent *Mtb* isolate HN878, possibly reflecting a virulence strategy employed by this strain (Koo *et al*, 2012). A study by Tailleux *et al* (2008) was the first to use microarray to determine the gene expression of both *Mtb* and the host cell (human macrophages and DCs) simultaneously. This study found cell-specific response to *Mtb*; for example macrophages but not DCs, upregulated genes encoding vesicular (v)-ATPase subunits (Tailleux *et al*, 2008). In addition, *Mtb* responded differently to macrophages and DCs; *Mtb* showed an increased stress response within DCs compared to macrophages, possibly reflecting the fact that DCs present a less permissive environment for *Mtb* growth compared to macrophages (Tailleux *et al*, 2008). Microarray has also been used to study the role of particularly signalling molecules in the transcriptional response of innate cells to *Mtb*. Transcriptional profiling of *Mtb*-infected macrophages deficient in the enzymes iNOS (Ehrt *et al*, 2001), the signalling molecule MyD88 (Shi *et al*, 2003) and the type I IFN receptor *Ifnar1* (Shi *et al*, 2005) has revealed important roles for these molecules in transcriptional regulation in response to *Mtb*. Notably, induction of a number of important genes including iNOS and IP10 was found to be MyD88-independent, but dependent on the IFNαβR, thus revealing important regulatory mechanisms that

occur in response to *Mtb* infection (Shi *et al*, 2005). Thus, microarray is a powerful tool for generating new insights into the response of innate immune cells to *Mtb*, and the mechanisms employed by *Mtb* to subvert this response. These studies are discussed in more detail in Chapter 3.

Chapter 2. Materials and Methods

2.1. Mice

C57Bl/6 (B6), B6 *Ifnar1*$^{-/-}$, B6 *Il10*$^{-/-}$, B6 *Tpl2*$^{-/-}$, B6 *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ and B6 *Tpl2*$^{-/-}$*Il10*$^{-/-}$ were bred and housed under specific pathogen free conditions at the MRC-NIMR under UK Home Office regulations. Female mice were used between 8-20 weeks of age.

2.2. Reagents

2.2.1. Cell culture medium

Cell culture medium used for all experiments was RPMI 1640 (Lonza) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Biosera), 0.05mM 2-mercaptoethanol (Sigma), 2mM L-glutamine (Lonza), 1mM sodium pyruvate (Lonza) and 10mM HEPES (Lonza). This is subsequently referred to as cRPMI.

2.2.2. Recombinant IFNs

Recombinant IFNβ was purchased from PBL and recombinant IFNγ was purchased from R&D systems. IFNβ was used at 2ng/ml unless otherwise indicated and IFNγ was used at 5ng/ml.

2.2.3. Inhibitors

The MEK1/2 inhibitor PD0325901 was purchased from Philip Cohen (University of Dundee, Scotland, UK) and reconstituted in Dimethyl sulfoxide (DMSO; Sigma). PD0325901 was used at a concentration of 0.1μM.

2.2.4. *Mtb*

All experiments using *Mtb* were carried out under bio-safety containment level 3 conditions. *Mtb* H37Rv was originally from the London School of Hygiene and Tropical Medicine (LSHTM) and grown at the MRC-NIMR. *Mtb* H37Rv was

grown by seeding either 100µl of frozen stock, or picking colonies on 7H11 plates, into 10ml of liquid 7H9 culture broth (BD Difco) supplemented with 0.05% Tween-80 (Sigma), 0.5% Glycerol (Sigma) and 10% oleic acid-albumin-dextrose catalase (OADC; BD Difco). 10ml cultures were placed in a rotor at 37ºC, rotating at 25rpm and growth was monitored by reading optical density at 600nm. When OD was approximately 1, 1ml of the rotating culture was added to 50ml 7H9 culture broth in a roller bottle, which was placed on a roller at 37ºC, at 2rpm. OD was monitored as above and when OD was approximately 1, *Mtb* was centrifuged for 20mins at 3000rpm to pellet cells, and the pellet was washed twice in phosphate-buffered saline (PBS) to remove any remaining media, and then resuspended in PBS. A slower spin was then carried out (10mins at 800rpm) to remove *Mtb* clumps, and the supernatant was aliquotted into freezing vials, 1ml per vial, and stored at -80°C. To determine the number of viable colony forming units (CFUs) of *Mtb* per tube in the stock, vials were thawed and serially diluted onto 7H11 plates supplemented with OADC. For each new stock, selected vials were plated onto blood agar plates, to ensure no contamination with other microorganisms. These vials were then thawed and used to infect macrophages as described below.

2.3. *In vitro* differentiation and infection of macrophages

2.3.1. Generation of murine macrophages from the bone marrow

To generate bone marrow-derived macrophages, bone marrow cells were flushed from the femurs and tibia of mice and seeded into bacterial plates (Sterilin) at $0.5x10^6$ cells/ml, in 8ml cRPMI containing 10% FCS and 20% L929 cell-conditioned medium (LCCM) which contains macrophage colony-stimulating factor (M-CSF). At day 4, 10ml fresh cRPMI containing 10% FCS and 20% LCCM was

added. At day 6, non-adherent cells were discarded, 5ml ice-cold PBS was added and plates were incubated at 4ºC for 15mins. Macrophages were then removed from the plate, pelleted by centrifugation at 1200rpm for 7mins and resuspended in cRPMI (5% FCS). Macrophages were counted and seeded into 24-well flat-bottomed tissue culture plates (Corning) at $1x10^6$ cells/ml, in a final volume of 1ml. For the microarray experiment presented in Chapter 3, macrophages were seeded into 6-well flat-bottomed tissue culture plates (Corning) at $1x10^6$ cells/ml, in a final volume of 3ml. Cells were rested overnight and on day 7 medium was removed, cells were washed once with PBS and infected as described below. Macrophage purity was found to be approximately 98% based on expression of CD11b and F4/80 by FACS analysis.

2.3.2. Infection of macrophages with *Mtb*

Macrophages were infected with *Mtb* at a multiplicity of infection (MOI) of 2:1 (bacteria: cell). *Mtb* was left in the wells until the supernatant or cells were harvested. The number of bacteria in the inoculum was determined by serial dilutions on 7H11 plates supplemented with 10% OADC.

2.3.3. Enumeration of intracellular *Mtb* in macrophages following infection

To determine the number of intracellular *Mtb* CFUs present in macrophages following infection, supernatants were harvested, macrophages were washed once with PBS to remove extracellular bacteria and 1ml of 0.2% saponin (Sigma) was added for 1hr at 37 ºC to lyse the cells. This suspension was then serially diluted and plated onto 7H11 plates supplemented with OADC, and colonies were counted after 14-16 days at 37ºC.

2.4. Cytokine quantification by ELISA

For quantification of cytokine concentrations in cellular supernatants, cell-free supernatants were harvested at the indicated time point post-infection and measured by enzyme-linked immune-adsorbent assay (ELISA). Commercially available kits were used for TNFα, IL-12p70 and IL-27 (eBioscience), IL-1β (R&D systems) and IFNβ (PBL), and were used according to the manufacturer's instructions. Matched antibody pairs were used for IL-10 and IL-12p40. Details of ELISAs used are shown in Table 2.1.

Cytokine	Standard starting concentration	Coating antibody	Detection antibody	Substrate	Detection limit
IL-10	10ng/ml	JES-2A5, 5µg/ml (DNAX)	SXC-1, 0.25µg/ml (BD biosciences)	TMB	40pg/ml
IL-12p40	50ng/ml	C15.6, 5µg/ml (DNAX)	C17.8, 0.5µg/ml (DNAX)	ABTS	50pg/ml
TNFα	10ng/ml	n/a	n/a	TMB	20pg/ml
IL-12p70	10ng/ml	n/a	n/a	TMB	20pg/ml
IL-1β	10ng/ml	n/a	n/a	TMB	20pg/ml
IFNβ	1000pg/ml	n/a	n/a	TMB	20pg/ml
IL-27	10ng/ml	n/a	n/a	TMB	20pg/ml

Table 2.1. Details of ELISAs used for quantification of cytokines

2.5. Protein analysis by Western blotting

For Western blot analysis, macrophages were rested overnight in cRPMI containing 1% FCS, and then stimulated with IFNγ at 5ng/ml in cRPMI with 0% FCS. At the indicated times post-stimulation, cells were lysed in RIPA buffer (0.1% SDS, 1% NP-40, 50 mM NaCl, 0.5% deoxycholate acid, 50 mM, Tris HCl, pH 8.0, 2 mM EDTA, 2 mM sodium-pyrophosphate, 50 mM sodium fluoride, 100 mM vanadate (all from Sigma-Aldrich), and complete EDTA-free protease inhibitor cocktail (Roche)). The concentration of protein in the lysates was determined by

BCA assay (ThermoScientific). Protein in the samples were then denatured by adding sodium dodecyl sulfate (SDS).

20ug of protein was loaded onto a 12.5% poly-acrylamide gel, which was run at 45mA for approximately 4hr to separate the proteins according to size. The protein in the gel was then transferred onto a methanol-activated membrane (Millipore) overnight at 180mA. The membrane was then blocked for 1hr at room temperature in 20ml PBS with 5% milk and 0.05% Tween-20 (Sigma). The membrane was then incubated with the primary antibody; total STAT1 (Cell Signalling; 1/1000 dilution overnight at 4ºC), Tyr701 phosphorylated STAT1 (Cell Signalling; 1/1000 dilution overnight at 4 ºC), or Actin as a loading control (Calbiochem; 1/5000 dilution for 2hr at room temperature), in 20ml PBS with 5% milk and 0.05% Tween-20. The secondary antibody (HRP-Conjugated Goat Anti-Rabbit IgG; Southern Biotech), at 1/2000 dilution in 20ml PBS with 5% milk and 0.05% Tween-20, was then added for 1hr at room temperature. The membrane was then developed using ECL (Millipore) and photographic paper.

2.6. RNA extraction and purification

2.6.1. Harvesting and purification of RNA

At indicated time point post-infection, macrophage supernatants were harvested and cells were washed once with PBS. 350µl RLT buffer (Qiagen) with 1% 2-mercaptoethanol was added to lyse cells. Lysates were stored at -80ºC until further processing. Lysates were homogenised using QIAshredder kits (Qiagen) and RNA was purified using RNeasy Mini Kits (Qiagen). An optional on-column DNA digestion step was performed using RNase-Free DNAse sets (Qiagen). All kits were used according to manufacturer's instructions. The concentration of purified RNA

was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Thermo Scientific). For RNA to be used for microarray analysis, RNA quality was assessed using an Agilent 2100 Bioanalyser (Agilent technologies). All samples had an RNA integrity number (RIN) of >9.3, which indicated high quality. Purified RNA was stored at -80°C for further processing.

2.6.2. Conversion of RNA to cDNA for qPCR analysis

RNA was reverse transcribed to cDNA using High Capacity cDNA Reverse Transcription kits (Applied Biosystems) using the master mix shown in Table 2.2.

Reagent	Company	Volume per reaction (final volume 20µl)	Final Concentration
RNA	N/A	10µl	N/A
RT buffer	Applied Biosystems	2µl	N/A
dNTPs	Applied Biosystems	0.8µl	4mM
Random Primers	Applied Biosystems	2µl	N/A
MultiScribe Reverse transcriptase	Applied Biosystems	1µl	2.5U/µl
RNAsin	Promega	0.5µl	1U/µl
Nuclease-free H_2O	Promega	3.7µl	N/A

Table 2.2. Reagents used for reverse transcription master mix

This mixture was incubated at 25ºC for 10mins, 37ºC for 2hrs then 85ºC for 5mins. Following conversion to cDNA, remaining RNA was digested by using RNAse H (Invitrogen), at a final concentration of 0.03U/µl, for 30mins at 37ºC. cDNA was then diluted to a final concentration of 5µg/ml and used for qPCR analysis as detailed below.

2.6.3. qPCR analysis

The expression of target genes was determined by qPCR (7900HT; applied Biosystems). Samples were placed in a 96-well optical reaction plate (Applied Biosystems) with volumes of reagents as shown in Table 2.3. Each plate also

contained a no-template control with 4.5ul H_20 replacing the cDNA, to control for contamination. The temperature and cycle number of the qPCR reactions is shown in Table 2.4. FAM-labelled Taqman primer probes were used to probe for specific genes. Following the reaction, the delta Ct (ΔCt) method was used to determine the expression value of the gene of interest (GOI) relative to a house-keeping gene, *Hprt* (hypoxanthine guanine phosphoribosyl transferase) using the following formula:

$$\Delta Ct\ GOI = 1.8^{(CtHprt - CtGOI)} \times 100000$$

Reagent	Company	Volume per reaction (final volume 10µl)	Final concentrations
cDNA	n/a	4.5µl	11.25ng/µl
Taqman universal PCR master mix	Applied Biosystems	5µl	n/a
Taqman primer probe	Applied Biosystems	0.5µl	900nM

Table 2.3. Reagents used for qPCR analysis

Temperature	Time	No. Cycles
50ºC	2mins	1
95ºC	10mins	1
95ºC/60ºC	15s/1min	40

Table 2.4. Temperatures and times used for qPCR reactions

Target Gene	Applied Biosystems catalogue number
Il10	Mm_00439616_m1
Il12a	Mm_00434165_m1
Il12b	Mm_00434174_m1
Tnf	Mm_00443258_m1
Il1b	Mm_00434228_m1
Ifngr1	Mm_00599890_m1
Ifna2	Mm_00833961_m1
Ifna5	Mm_00833976_m1
Ifna6	Mm_0170358_s1
Ifnb1	Mm_00439552_s1
Fos	Mm_00487425_m1
Egr1	Mm_00656724_m1
Jmjd3	Mm_01332680_m1
Ier3	Mm_00519290_g1
Dusp1	Mm_00457274_g1
Dusp5	Mm_01266106_m1
Hprt	Mm_00446968_m1

Table 2.5 Taqman primer probes used for qPCR

2.7. Microarray processing and data analysis

2.7.1. Amplification

For microarray analysis, 300ng of total RNA was reverse transcribed to cDNA, purified and used as a template for *in vitro* transcription to generate biotin labelled, antisense complementary RNA (cRNA) target molecules, using the Illumina TotalPrep-96 RNA Amplification Kit (Illumina) according to the manufacturer's instructions. The concentration of purified cRNA was determined using a NanoDrop 1000 spectrophotometer (NanoDrop Products, Thermo Scientific).

2.7.2. Hybridisation

750ng of biotin-labelled cRNA was hybridized overnight to Illumina Mouse WG-6 V2.0 Beadchip arrays (Illumina). The arrays were then washed, blocked,

stained and scanned on an Illumina BeadStation 500 following the manufacturer's protocols. Illumina BeadStudio software (Illumina) was used to generate signal intensity values from the scans.

RNA amplification for the microarray data presented in Chapter 3 was performed by Dr Christine Graham of the Division of Immunoregulation, MRC National Institute for Medical Research, Mill Hill, London, UK. Hybridisation of cRNA to arrays, scanning and pre-processing for the microarray data presented in Chapter 3 was performed by Quynh-Anh Nguyen, Microarray Core Facility, Baylor Institute for Immunology Research, Dallas, Texas. RNA amplification and hybridisation for the microarray data presented in Chapters 4, 5 and 6 was carried out by Dr Harsha Jani of the Division of Systems Biology, MRC National Institute for Medical Research, Mill Hill, London, UK.

2.7.3. Microarray data analysis

2.7.3.1. Data pre-processing, normalisation and removal of undetectable transcripts

Illumina BeadStudio software was used to subtract background from signal intensity values. GeneSpring GX version 11 (Agilent Technologies) was used to perform further normalisation and data analysis. The following pre-processing and normalisation steps were applied to all microarray data presented. A threshold signal value of 10 was set, and all signal intensity values less than 10 were set to equal 10, to remove noise due to low values. Signal values were then log-transformed to the base 2. Per-chip normalisation was applied using the 75^{th} percentile shift algorithm. This normalisation reduces differences in gene expression due to technical variations between different samples within a chip, and between chips, such as differences is

labelling or hybridisation (Quackenbush, 2002). Per-gene normalisation was then applied by dividing the signal value for each gene in each sample by the median of the signal value for that gene across all samples. Prior to further analysis, all transcripts were filtered to select transcripts that were significantly detectable from background: those called 'present' in 100% of any one group of replicates ($p<0.01$).

2.7.3.2. Fold-change and statistical analysis

Following the removal of undetectable transcripts, fold-change and statistical analyses were applied to generate lists of differentially expressed transcripts, using GeneSpring. The specifics of the analyses carried out to generate lists of differentially expressed transcripts are presented in the relevant results section and figure legend. Fold change filters retained transcripts with greater than 2-fold changes in expression between conditions. For statistical analyses, two-way ANOVA ($p<0.05$) with Benjamini Hochberg FDR multiple testing correction was used.

2.7.3.3. Hierarchical and *k*-means clustering

Lists of differentially expressed transcripts generated by fold-change and statistical analysis were subjected to hierarchical clustering. This technique constructs a vertical dendrogram where transcripts with similar expression profiles across the condition are grouped together, and is useful in identifying patterns within the gene expression dataset (Quackenbush, 2002). The dendrogram is then displayed with gene expression shown as a red-blue heat map, with red indicating upregulation, blue indicating downregulation and yellow no change. For all hierarchical clustering a Pearson centred distance metric was used, as this clusters transcripts based on the expression profile of transcripts over the different samples, rather than the magnitude

of gene expression which is used by Euclidian-based distance metric (Quackenbush, 2002). This was because transcripts with similar expression patterns, e.g. transcripts with expression at a particular time point, are likely to be co-regulated regardless of if one transcript is induced to a higher magnitude. In most cases, Pearson centred clustering was combined with complete linkage rule, which tends to generate compact clusters of a similar size (Quackenbush, 2002).

A second form of clustering, *k*-means clustering, was also applied to lists of differently expressed transcripts, again using a Pearson centred distance metric. This method separates the transcripts into a defined number of clusters (k) which can then be analysed in more detail (Quackenbush, 2002).

2.7.3.4. Functional analysis using IPA and GO analysis

Ingenuity Pathways Analysis (IPA) (Ingenuity Systems, Inc., Redwood, CA, USA, www.ingenuity.com) was used to determine signalling pathways associated with clusters of differentially expressed transcripts. IPA is a database containing more than 9,800 human, 7,900 mouse and 5,000 rat genes, which is manually curated with gene associations generated from over 200,000 full text scientific articles (Calvano *et al*, 2005). Gene lists generated as described above were uploaded into IPA, and Fisher's exact test, with Benjamini Hochberg FDR multiple testing correction was used to determine statistically significant overlap between gene lists and canonical IPA signalling pathways. In this study, an interactions was considered significant if $p<0.01$. IPA was also used to overlay gene expression data onto a canonical pathway, with common genes highlighted either in red, if over-represented in the dataset, or blue, if under-represented.

Further functional analysis was carried out using gene ontology (GO) analysis in GeneSpring. This analysis uses the GO database, where genes are assigned ontologies relating to their molecular functions, associated biological processes and cellular location (Harris *et al*, 2004). Lists of genes can then be analysed with regard to their associated GO ontologies, and significant associations with particular terms can be calculated.

2.8. Statistical analysis

Statistical analysis on experimental data was carried out using PRISM software. T-test, 1-way ANOVA and 2-way ANOVA were used as indicated in the figure legend, with a significant result taken with a p-value of less than 0.05.

Chapter 3. The macrophage transcriptional response to *Mtb*

3.1. Background

Macrophages are the immune system's first line of defence against pathogenic microorganisms, populating key sites of the body where interactions with the outside environment occur, and engulfing and destroying foreign material, including bacteria. Macrophages also produce cytokines and chemokines which attract and activate other immune cells (Medzhitov and Horng, 2009). Alveolar macrophages in the lung are the first cells to encounter *Mtb* following infection, but *Mtb* has evolved mechanisms to survive within macrophages, generating a niche in which to replicate (Pieters, 2008). Alveolar macrophages, and recruited macrophages, are therefore a major reservoir for *Mtb* throughout the course of infection (Wolf *et al*, 2007). The interactions between *Mtb* and macrophages are therefore critical in determining the outcome of infection.

Macrophages recognise pathogens through pattern recognition receptors (PRRs), which detect conserved features unique to microorganisms. *Mtb* is recognised by macrophages through Toll-like receptors (TLRs), C-type lectins including dectin-1, DC-SIGN and the mannose receptor (MR), and the Nod-like receptor NOD2 (Kleinnijenhuis *et al*, 2011). Recognition of a pathogen by PRRs on macrophages triggers many signalling pathways, resulting in the activation of transcription factors such as NF-κB, and the induction and repression of hundreds of genes involved in the antimicrobial response (Jenner and Young, 2005; Medzhitov and Horng, 2009). A thorough understanding of how the macrophage response is regulated will lead to new insights into the factors required to protect against pathogens such as *Mtb*, and how these pathogens are able to subvert this response to favour their own growth.

Given the complexity of the macrophage response to pathogens or pathogen derived products, microarray is an ideal tool to study this process, as it allows the user to determine the expression of thousands of genes in an unbiased manner. A number of studies have used microarray to study the response of human (Nau *et al*, 2002; Chaussabel *et al*, 2003; Wang *et al*, 2003; Tailleux *et al*, 2008) or mouse (Ehrt *et al*, 2001; Shi *et al*, 2003; Shi *et al*, 2005; Koo *et al*, 2012) macrophages to *Mtb*. The major findings from these studies, and the cell types, time points and stimuli used are shown in Table 3.1.

As shown in Table 3.1, these studies have led to important insights into the host pathogen interactions that occur in *Mtb*-infected macrophages. For example, Shi *et al* (2005) found that a number of immunologically important genes were induced by *Mtb* via type I IFN and STAT1 signalling, independently of TLRs, at 4hr post-infection. A more recent study by Tailleux *et al* (2008) analysed the response of human macrophages and DCs to *Mtb*, and additionally profiled *Mtb* gene expression within these two cell types. This revealed cell-specific responses to *Mtb* by macrophages and DCs, with macrophages upregulating genes involved with phagosome acidification and vesicle trafficking (Tailleux *et al*, 2008).

However, previous microarray studies of *Mtb*-infected macrophages have tended to use a limited number of time points (Table 3.1), and so the dynamics of the macrophage response to infection was not assessed (Ehrt *et al*, 2001; Chaussabel *et al*, 2003; Shi *et al*, 2003; Wang *et al*, 2003; Shi *et al*, 2005; Tailleux *et al*, 2008; Koo *et al*, 2012). In addition, the majority of these studies did not analyse macrophage gene expression at time points earlier than 4hr post-infection (Ehrt *et al*, 2001; Chaussabel *et al*, 2003; Shi *et al*, 2003; Wang *et al*, 2003; Shi *et al*, 2005; Tailleux *et al*, 2008; Koo *et al*, 2012) (Table 3.1). Although Nau *et al* (2002) analysed the

response of human macrophages to *Mtb* at 1hr and 2hr post-infection, the primary aim of this study was to compare the common macrophage response to various bacterial pathogens, and the response to *Mtb* was not analysed in detail. Microarray studies of LPS stimulated macrophages have found that gene expression can change rapidly following treatment, with clusters of upregulated genes observed as early as 1hr post-stimulation (Gilchrist *et al*, 2006; Ramsey *et al*, 2008). These genes are likely to regulate the macrophage response at later time points, and could play an important role in the immune response.

Here we carried out a microarray analysis of *Mtb*-infected macrophages over an extended time course, including both early and late time points. We found the macrophage response to *Mtb* to be highly dynamic, with many clusters of genes induced or repressed at different time points. This included early clusters of genes, which were induced at 30mins to 1hr post-infection. Analysis of these clusters by ingenuity pathway analysis (IPA) and gene ontology (GO) identified clusters of genes involved with a wide range of functions, some of which would protect the host against *Mtb* infection, and others that could be a result of *Mtb* virulence strategies to subvert the protective immune response. This illustrates the complexity of *Mtb*-macrophage interactions, and provides a framework for future analyses.

Reference	Cell type	*Mtb* strain	Time points	Primary Findings
Ehrt *et al*, 2001	Murine macrophages	Clinical isolate 1254	24hr	• Synergy between *Mtb* and IFNγ in induction of inflammatory genes, including iNOS • iNOS and NADPH oxidase found to be important transcriptional regulators in response to *Mtb*
Nau *et al*, 2002	Human macrophages	Erdman	0, 1, 2, 6, 12, 24hr	• Defined common macrophage activation program in response to *Mtb* and various bacteria, including cytokines, chemokines, receptors and transcription factors • Response to *Mtb* not analysed in detail
Chaussabel *et al*, 2003	Human macrophages and DCs	DNS	16hr	• Compared macrophage and DC response to *Mtb* and other intracellular pathogens • Found common and pathogen-specific cluster of genes • Type I IFN inducible and MHC class I related genes induced by *Mtb* and *Toxoplasma gondii* in DCs and macrophages • Cytokines, chemokines and NF-κB related genes induced by *Mtb* in macrophages and DCs
Shi *et al*, 2003	Murine macrophages	Clinical isolate 1254	24hr	• *Mtb* transcriptional response largely MyD88-independent • MyD88 required for macrophage activation in response to IFNγ
Wang *et al*, 2003	Human macrophages	Erdman	4, 12, 24, 48hr	• Found upregulation of cytokines, chemokines, proteasome components and STAT1
Shi *et al*, 2005	Murine macrophages	Clinical isolate 1254	24hr	• Induction of several immunologically important genes, including RANTES, IRG1, IP10 and iNOS independent of MyD88 but dependent on type I IFN and STAT1

Tailleux *et al*, 2008	Human macrophages and DCs	H37Rv	4, 18, 48hr	• Cell-specific responses to *Mtb* identified • NADPH oxidase, phagosome acidification and intracellular trafficking regulated genes more strongly induced in macrophages • Type I IFN and TLR related genes more strongly induced in DCs • Profiling of *Mtb* gene expression found cell-specific adaptations to macrophages and DCs, with replication related genes upregulated in macrophages, and stress response genes upregulated in DCs
Wu *et al*, 2012	Human THP-1 macrophage-like cell-line	11 W-Beijing strains and H37Rv	4, 18, 48hr	• Similar response to W-Beijing strains compared to H37Rv • Core response included many cytokines, chemokines and receptors • Found induction of many IFN-inducible genes, which overlapped with IFN-signature identified by Berry *et al* (2010)
Koo *et al*, 2012	Murine macrophages	HN878 and CDC1551	6 and 24hr	• Immune response genes induced by CDC1551 but not HN878 at 6hr post-infection • Genes involved in lipid metabolism upregulated in response to HN878 but not CDC1551 at 24hr post-infection

Table 3.1. Summary of previous microarray studies of innate immune cells infected with *Mtb*

3.2. Results

3.2.1. The macrophage response to *Mtb* is temporally regulated

To gain a global understanding of the transcriptional response of macrophages to *Mtb* infection, we began by using transcriptional profiling to study the global macrophage response to *Mtb* over an extended time-course, including both early and late time points. Macrophages were derived from the bone marrow of wild-type (WT) mice, and were infected with the H37Rv strain of *Mtb* (which is used throughout this study) at a multiplicity of infection (MOI) of 2:1. This MOI was chosen based on preliminary experiments showing that an MOI of 2:1 activated macrophages to produce cytokines, but without causing significant cell death, and is used throughout this study. RNA was harvested from infected and uninfected macrophages at 0hr, , 30mins, 1hr, 3hr, 6hr and 24hr post-infection. Purified total RNA was amplified, labelled and hybridised to Illumina Mouse WG-6 V2.0 Beadchip arrays, as described in the Materials and Methods, with duplicate biological samples for each condition. In addition, selected samples were run independently for a second time, and similar results were observed (data not shown). Background subtraction was carried out using BeadStudio (Illumina), and further analysis was carried out using GeneSpring (Agilent Technologies).

The 42581 analysed transcripts were filtered first to select transcripts that were significantly detectable from the background: those called ‘present’ in 100% of any 1 group of 2 triplicates. This left 15018 transcripts. To gain an overview of the dataset, the 26 samples were subjected to unsupervised clustering, using a Euclidian distance metric with complete linkage, based on the expression of the 15018 detectable transcripts. This technique clusters samples based on how similar they are

to each other, and thus shows the overall trends within the dataset (Quackenbush, 2002).

Figure 3.1 shows that the 26 samples were separated into two groups by unsupervised clustering. One group contained *Mtb*-infected samples at 3hr, 6hr and 24hr, while the second larger group contained all uninfected samples and *Mtb*-infected samples at 15mins, 30mins and 1hr. This suggests that the major *Mtb*-induced transcriptional changes occur at 3hr, 6hr and 24hr post-infection, as these samples cluster separately from the uninfected controls. The *Mtb*-infected samples at 15mins and 30mins cluster together with the uninfected controls (Figure 3.1), suggesting minimal changes have occurred in response to *Mtb* at these early time points. However, although the 1hr *Mtb*-infected samples cluster in the same groups as the uninfected controls, they are on a separate branch, suggesting that some changes are occurring at this time point (Figure 3.1).

To investigate further, two filters were applied to the 15018 detectable transcripts, designed to retain only transcripts that changed in response to *Mtb* infection over time. First, transcripts were retained if their expression changed by greater than 2-fold in response to *Mtb* at any time point, relative to the mean of the uninfected controls at that time point. Second, transcripts were retained if they were statistically different with regard to infection and time, as determined by two-way ANOVA ($p<0.05$) with Benjamini Hochberg FDR multiple testing correction. This left 6479 *Mtb*-regulated transcripts. To study the expression profile of these transcripts over time, they were clustered hierarchically using Pearson centred distance metric and complete linkage (Figure 3.2). Hierarchical clustering groups transcripts with similar expression profiles into clusters, generating a global overview of the gene expression in a dataset (Butte, 2002; Do and Choi, 2008). A

Pearson based distance metric was used as it clusters transcripts on the basis on the trend of their expression profile, rather than the magnitude of the change, which is informative as similar trends of expression can indicate common regulation and biological function (Do and Choi, 2008).

As predicted by unsupervised clustering of the samples (Figure 3.1), the majority of transcriptional changes in response to *Mtb* occurred at 3hr, 6hr and 24hr post-infection (Figure 3.2). No transcriptional changes were visible at 15mins post-infection (Figure 3.2). However, a small number of transcripts were upregulated at 30mins, and this increased slightly at 1hr post-infection (Figure 3.2), showing that certain genes are induced rapidly in macrophages following *Mtb* infection. This early response is likely to be important in inducing and regulating the later response of macrophages to *Mtb*. It was striking that the transcripts showed considerable heterogeneity in their expression profiles (Figure 3.2). Some clusters of transcripts remained upregulated or downregulated over relatively long periods of time, e.g. between 3hr and 24hr, whereas others were regulated only at one time point (Figure 3.2).

Taken together, this analysis shows that the macrophage response to *Mtb* occurs predominantly between 3hr and 24hr post-infection, but with small numbers of transcripts induced early (30mins-1hr) following infection. In addition, this response was highly dynamic, with groups of transcripts changing expression with varying kinetics. This illustrates the importance of using multiple time points, including early time points, to study the temporal response of immune cells to infection, as important transcriptional changes may be missed if few time points are used.

3.2.2. *k*-means clustering separates the macrophage response to *Mtb* into 24 clusters, based on their expression profiles

Figure 3.2 shows that *Mtb*-induced transcription is complex, with many discrete clusters being up- or downregulated with varying kinetics. Clusters of transcripts with similar profiles are likely to be involved in shared processes, and to be regulated by common pathways or transcription factors (Do and Choi, 2008). In order to further study the regulation and function of these clusters, it was necessary to separate them into lists of transcripts which could be analysed in more detail. To achieve this, we used GeneSpring to separate the 6479 transcripts into clusters using *k*-means clustering. *k*-means clustering separates transcripts into a pre-defined number of clusters based on their expression profile (Butte, 2002). We used *k*-means clustering to separate the 6479 *Mtb*-regulated transcripts in 24 clusters, again using a Pearson centred distance metric. The 24 clusters varied in size, with the smallest (cluster 15) containing 51 transcripts and the largest (cluster 7) containing 707 transcripts. Complete lists of the transcripts are presented in section 3.5 at the end of this chapter.

The expression profiles of the 24 clusters are shown in Figure 3.3. Of the 24, 17 clusters contained transcripts that were upregulated in response to *Mtb* infection and seven were downregulated, reflecting the higher number of upregulated transcripts and the greater heterogeneity of their expression (Figure 3.3). As predicted from Figure 3.2, the clusters varied enormously in their kinetics of induction or repression over time. Of those upregulated in response to *Mtb*, two showed changes at 1hr post-infection; cluster 8, which remained upregulated throughout the course of infection, and cluster 15, which was induced only transiently and returned to baseline by 3hr post-infection (Figure 3.3). The 15 other

upregulated clusters also showed considerable variability in their expression. Cluster 1 (3hr), cluster 9 (6hr) and clusters 14 and 24 (24hr) were only upregulated at one time point following *Mtb* infection (Figure 3.3). Others remained upregulated over two time points; clusters 0, 2 and 20 at 6hr-24hr post-infection and clusters 5 and 12 at 3hr-6hr post-infection (Figure 3.3). Finally, clusters 4, 6, 11, 19, 22 and 23 were induced by *Mtb* and remained upregulated at 3hr, 6hr and 24hr post-infection, although the peak and magnitude of expression varied between these clusters (Figure 3.3). The downregulated clusters showed similar heterogeneity, with cluster 3 downregulated only at 24hr post-infection, cluster 10 at 6hr and 24hr post-infection, clusters 7 and 17 at 3hr and 6hr post-infection and clusters 16 and 18 at 3hr, 6hr and 24hr (Figure 3.3). In summary, the 6479 genes making up the macrophage response to *Mtb* can be separated into 24 clusters of induced and repressed genes, with large dynamic and temporal variability in their expression profiles.

3.2.3. *Mtb*-regulated gene clusters are associated with a variety of functions and signalling pathways

Having separated the transcriptional response of macrophages to *Mtb* into 24 clusters, we next investigated the functions of these clusters, and the signalling pathways that regulate their expression. Two approaches were used; gene ontology (GO) analysis (using GeneSpring), which gives broad information as to the potential functions of transcript lists, and ingenuity pathway analysis (IPA) which gives more specific information on the signalling pathways associated with transcript clusters. Each of the 24 clusters were analysed by GO analysis and IPA, with the top 5 significant overlaps for each analysis shown ($p<0.01$) (Figure 3.4.1-3.4.4). Where significant associations were found, these were confirmed by literature searching of

the relevant transcripts, under the column "literature associations". In addition, function or signalling associations not picked up by IPA or GO analysis, but suggested by published literature, are shown in this column.

Of the 24 clusters, eight showed no significant overlap with either GO terms or IPA signalling pathways (clusters 0, 1, 3, 6, 14, 18, 20 and 21) (Figure 3.4.1-3.4.4). The remaining 16 clusters showed overlap with a wide range of biological processes and signalling pathways (Figure 3.4.1-3.4.4). These are discussed below selected clusters hierarchically clustered and visualised with a heat map to show the gene expression in more detail, with the relevant genes in each cluster indicated (Figures 3.5-3.17).

3.2.3.1. Early clusters are associated with NF-κB and ERK MAP kinase signalling

A small number of transcripts were upregulated at 30mins to 1hr following *Mtb* infection (Figure 3.2). *k*-means clustering separated these transcripts into two clusters with differing expression profiles (Figure 3.3). Cluster 8 contained transcripts that were induced early and remained upregulated throughout the infection, whereas transcripts in cluster 15 were induced only transiently, and were rapidly downregulated after peaking at 1hr post-infection (Figure 3.3).

GO analysis of cluster 8 showed a strong association with terms involved with cytokines and chemokines, such as "cytokine activity" and "chemokine activity" (Figure 3.4.2). This was due to the presence of the cytokines *Il1b* and *Tnf* and the chemokines *Ccl2*, *Ccl3*, *Ccl4*, *Ccl7*, *Cxcl1* and *Cxcl2* (Figure 3.5). These cytokines and chemokines function to mediate inflammation and stimulate the recruitment of further immune cells to the site of infection, and are essential for a

protective response to *Mtb* infection (Flynn *et al*, 1995; Peters *et al*, 2001; Mayer-Barber *et al*, 2011). The rapid production of these cytokines and chemokines by *Mtb*-infected macrophages is likely to lead to the influx of further immune cells into the lung, and lead to the formation of the granuloma. Cluster 8 also includes several transcription factors including *Irf1*, *Tgif1*, *Maff*, *Atf3* and *Junb*, suggesting that this early response also leads to subsequent waves of transcription. Cluster 8 also contains the immunosuppressive cytokine *Il10* (Figure 3.5). IL-10 can inhibit the production of IL-1β and TNFα from macrophages (Fiorentino *et al*, 1991a; Fiorentino *et al*, 1991b; Moore *et al*, 2001) suggesting that this early pro-inflammatory macrophage response is regulated to prevent host damage.

The IPA signalling pathways that were associated with cluster 8 included "IL-10 signalling" and "IL-17 signalling" (Figure 3.4.2). However, induction of these pathways would require autocrine signalling, and this is unlikely given the extremely rapid induction of the transcripts in cluster 8. We therefore investigated the regulation of these transcripts in more detail by using IPA to determine the transcriptional regulators associated with the transcripts in cluster 8. As shown in Table 3.1, the most significant transcription factors associated with this cluster were the NF-κB family of transcription factors, which are known to induce pro-inflammatory genes downstream of a wide variety of signals including TLRs (Vallabhapurapu and Karin, 2009). Genes in cluster 8 linked to NF-κB family members included *Il1b*, *Tnf*, *Ccl2*, *Ccl4*, *Ccl7* and *Cxcl2* (Figure 3.5). These data therefore suggest that NF-κB is rapidly activated in *Mtb*-infected macrophages, and this stimulates the production of vital cytokines and chemokines.

Transcriptional Regulator	P-value of overlap
NF-κB (complex)	$1.95x10^{-13}$
STAT3	$2.20x10^{-11}$
EGR1	$3.66x10^{-10}$
NFKB1	$3.66x10^{-10}$
GLI2	$1.20x10^{-8}$

Table 3.2. Transcriptional regulators associated with cluster 8 by IPA analysis

Cluster 15, which was induced rapidly but transiently in *Mtb*-infected macrophages (Figure 3.6A), was associated with GO terms involved with gene regulation, such as "Regulation of transcription from RNA polymerase II promoter" and "Regulation of gene expression" (Figure 3.4.3). This was due to the presence of the transcription factors *Fos*, *Fosb*, *Fosl2*, *Myc*, *Jun* and *Egr1* (Figure 3.6B). Early induction of these transcription factors would be expected to regulate transcription at later time points; for example, c-Fos (encoded by *Fos*) is known to regulate the production of IL-10, IL-12 and IFNβ downstream of TLR signalling (Dillon *et al*, 2004; Kaiser *et al*, 2009). In addition, cluster 15 included the histone H3K27 demethylase *Jmjd3* (Agger *et al*, 2007), suggesting that chromatin remodelling may occur in response to *Mtb*.

We noticed that cluster 15 contained many genes that are known targets of the ERK MAP kinase signalling pathway. This included the transcription factors *Fos* (Gineitis and Treisman, 2001; Kaiser *et al*, 2009) and *Egr1* (Gineitis and Treisman, 2001; Harada *et al*, 2001), the phosphatases *Dusp1* (Chen *et al*, 2002) and *Dusp5* (Kucharska *et al*, 2009), *Ier3* (Garcia *et al*, 2002; Hamelin *et al*, 2006) and the histone H3K27 demethylase *Jmjd3* (Lin *et al*, 2011b) (Figure 3.6B). To validate the early expression of these genes, and the microarray data as a whole, qPCR was carried out using the same RNA as was used for microarray analysis. Figure 3.6C

shows that *Fos*, *Egr1*, *Dusp1*, *Dusp5*, *Ier3* and *Jmjd3* were induced rapidly in macrophages infected with *Mtb*, but rapidly returned to baseline, confirming the results observed using microarray analysis.

These results suggest that the ERK MAP kinase pathway is rapidly activated in macrophages in response to *Mtb* infection, and that this pathway leads to the induction of a transient cluster of transcription factors and transcriptional regulators. The ERK MAP kinase pathway is therefore likely to have an important bearing on the subsequent macrophage response. This prompted us to further investigate the role of the ERK MAP kinase pathway in *Mtb*-infected macrophages, and the results are presented in Chapter 5.

3.2.3.2. Further pro-inflammatory genes are contained in cluster 23

Like cluster 8, GO analysis of cluster 23 also showed a significant association with pro-inflammatory functions, including "myeloid leukocyte activation" and "inflammatory response" (Figure 3.4.4). This cluster, however, was expressed later than cluster 8, with expression first induced at 3hr post-infection, compared to 30mins-1hr post-infection for cluster 8, showing that the inflammatory response occurs in waves (Figure 3.7). This may suggest that different pathways and transcription factors regulate these two clusters. Cluster 23 contained the cytokines *Il6*, *Il15* and *Il27*, the intracellular receptor *Nod2*, the cell surface receptor *Cd40*, the acute phase protein *Saa3* (serum amyloid A3) and the TLR adaptor molecules *Myd88* and *Ticam1* (TRIF) (Figure 3.7). This cluster would therefore lead to enhanced inflammation, but also lead to more specific responses such as the activation of NK cells (IL-15) and the mediation of T cell activation of macrophages (*Cd40*), and illustrates the diversity of functions carried out by macrophages.

3.2.3.3. Clusters associated with iNOS induction

An important part of the macrophage response to *Mtb* is the production of reactive nitrogen intermediates (RNIs) by the enzyme iNOS, encoded by *Nos2* (Chan *et al*, 1992; Chan *et al*, 1995). Two clusters were associated with the induction of RNIs by IPA analysis; cluster 12 showed significant overlap with the IPA pathway "role of nitric oxide and reactive oxygen species in macrophages" (Figure 3.4.3). and cluster 23 showed overlap with "production of nitric oxide and reactive oxygen species in macrophage" (Figure 3.4.4). However, the transcripts found to be involved with RNI induction, *Rap1a*, *Rhou*, *Rhog*, *Rap1b*, *Jak2* and *Pik3r5*, are involved in many different signalling pathways. For example, *Rap1a*, *Rhou*, *Rhog* and *Rap1b* are small GTPases known to regulate a large number of biological process including cytoskeleton remodelling and cell-proliferation (Kaibuchi *et al*, 1999). This illustrates a caveat in the use of gene-list interpretation software such as IPA, and shows that associations should be confirmed by literature searching. However, the enzyme *Nos2* (iNOS) was strongly induced by *Mtb* at 3hr post-infection, forming part of cluster 4 (Figure 3.8), suggesting that macrophages do produce RNIs in response to *Mtb*.

3.2.3.4. Genes in cluster 4 are associated with the MHC class I antigen processing pathway and ubiquitination

Cluster 4 contains 372 transcripts which are upregulated at 3-6hr post-infection and remain upregulated up to 24hr post-infection (Figure 3.4.1; Figure 3.8). GO analysis of the transcripts in cluster 4 found a strong association with the GO terms "cytosol", "cytoplasm" and "cytoplasmic part", suggesting involvement with cytoplasmic processes (Figure 3.4.1). In addition, GO analysis found an overlap with

the GO term "proteasome complex". This was due to the presence of numerous genes encoding subunits of the proteasome, including *Psma4*, *Psma7*, *Psmb2*, *Psmb3*, *Psmb5*, *Psmb7*, *Psmd1*, *Psmd14* and *Psme1* (Figure 3.8). The proteasome is a large, multi-subunit protease complex found in the cytoplasm, and functions to degrade proteins into short peptides (Vyas *et al*, 2008). The peptides produced are loaded onto MHC class I molecules and presented on the cell surface, allowing recognition of antigens by $CD8^+$ T cells, which can lead to killing of the infected cell through apoptosis (Banchereau and Steinman, 1998). Induction of the proteasome genes in *Mtb*-infected macrophages suggests that the MHC class I pathway may be activated in response to infection. In support of this, cluster 4 also contained the MHC class I molecules, *H2-K1* and *H2-M2*; and *Erap1*, an aminopeptidase which further processes peptides in the endoplasmic reticulum (ER) prior to their loading onto MHC class I molecules (Figure 3.8) (Saric *et al*, 2002).

Analysis of cluster 4 by IPA found a highly significant overlap with "protein ubiquitination pathway" (Figure 3.4.1). Ubiquitin is a small protein that is covalently attached to other proteins by ubiquitin ligases, a process which regulates numerous cellular processes . The association between cluster 4 and ubiquitination was in part due to the proteasome subunits discussed above, as ubiquitination targets proteins to the proteasome for degradation (Malynn and Ma, 2010). However, cluster 4 also contained several ubiquitin ligases, including *Smurf1*, *Ube2m* and *Ube2k* (Figure 3.8). This suggests that in response to *Mtb* infection, ubiquitin ligases are activated and this leads to the degradation of proteins via the proteasome. This may play an important role in the macrophage response, as ubiquitination has been shown to regulate many innate immune processes and signalling pathways, such as NF-κB signalling (Malynn and Ma, 2010).

3.2.3.5. Cluster 19 is associated with apoptosis

Cluster 19, consisting of 212 transcripts induced from 3hr post-infection by *Mtb* (Figure 3.9), was strongly associated with GO terms involving apoptosis and cell-death (Figure 3.4.4). These were "programmed cell death", "apoptosis", "cell death" and "death" (Figure 3.4.4). Genes associated with these terms were the Bcl-2 family members *Bcl2a1b, Bcl2a1c, Bcl2a1d* and *Bcl3*; the caspase *Casp4*; the death receptor *Fas*, the NLR family member *Nlrp3*; and the signalling molecules *Ripk2* and *Traf2* (Figure 3.9).

However, cluster 19 included genes with both pro-apoptotic and anti-apoptotic function. The pro-apoptotic genes included *Fas*, a "death-receptor" which upon binding of FasL initiates apoptosis via its cytoplasmic death domain, leading to the activation of caspase 8. Caspase 8 (encoded by *Casp8*) was also induced by *Mtb*, although it was found in cluster 2, as its expression was induced at 6hr and 24hr post-infection. Cluster 19 also included *Bcl2l11*, which encodes for the protein Bim, a Bcl2 family member involved in the initiation of apoptosis (Figure 3.9) (Borner, 2003). Induction of these genes suggested that macrophages undergo apoptosis in response to *Mtb* infection, which has been shown to be an important process as apoptosis of macrophages reduces the spread of *Mtb* within the lungs, and leads to an enhanced T cell response via cross-priming of $CD8^+$ T cells (Behar *et al*, 2011).

Anti-apoptotic genes in cluster 19 included *Birc2* and *Birc3*, members of the inhibitors of apoptosis (IAP) family of proteins (Figure 3.9). IAP proteins inhibit the activity of caspases, the enzymes responsible for mediating apoptosis (Siegel, 2006). Apoptosis has an important role in limiting the viability and spread of *Mtb*, and leads to cross-priming of the $CD8^+$ T cell response (Behar *et al*, 2011). As a result, virulent *Mtb* strains, including H37Rv, are known to actively prevent apoptosis, leading

instead to necrosis which promotes dissemination of *Mtb* (Divangahi *et al*, 2010; Behar *et al*, 2011). Induction of anti-apoptotic genes such as *Birc2* and *Birc3* may therefore represent a virulence strategy employed by *Mtb* to prevent apoptosis.

3.2.3.6. Association of cluster 9 with steroid biosynthesis

Both GO analysis and IPA analysis strongly linked cluster 9 to the synthesis of steroids (Figure 3.4.2). Transcripts in cluster 9 were upregulated specifically at 6hr following *Mtb* infection (Figure 3.10). GO terms significantly associated with cluster 9 included "steroid biosynthetic process", "sterol biosynthetic process" and "cholesterol biosynthetic process". Sterols are a subgroup of steroids, and cholesterol is a sterol, strongly suggesting that genes involved in steroid biosynthesis are upregulated in response to *Mtb* infection. In support of this, IPA showed a highly significant overlap between genes in cluster 9 and the pathway "biosynthesis of steroids" (Figure 3.4.2). Genes in cluster 9 linked to steroid biosynthesis included the enzymes *Fdps* (farnesyl disposphate synthase), *Mvd* (mevalonate decarboxylase), *Pmvk* (phosphomevalonate kinase), *Nhdsl* (pterol-4-alpha-carboxylate 3-dehydrogenase), *Stard* (Star-related lipid transfer protein 5) and *Lss* (Lanosterol synthase) (Figure 3.10). Taken together, these results show that macrophages activate pathways involving the biosynthesis of steroids at around 6hr post-infection with *Mtb*. This is relevant, as several studies have shown that cholesterol is vital for the entry of *Mtb* into macrophages, and its persistence within macrophages *in vivo* during latent infection (Gatfield and Pieters, 2000; Van der Geize *et al*, 2007; Pandey and Sassetti, 2008). Thus, production of cholesterol and other steroids by macrophages may favour the intracellular survival of *Mtb*.

Cluster 9 also contained genes linked to the proteasome and proteasome ubiquitination pathways, similarly to genes in cluster 4. GO analysis showed significant overlap between cluster 9 and "proteasome complex", and IPA analysis showed significant overlap with "protein ubiquitination pathway" (Figure 3.4.2). Similarly to cluster 4, cluster 9 included several genes encoding subunits of the proteasome; *Psmb10*, *Psma5*, *Psme2*, *Psmd8* and *Psmb6* (Figure 3.10). Genes of the proteasome are therefore induced with different kinetics in *Mtb*-infected macrophages, with those in cluster 4 induced from 3hr, 6hr and 24hr post-infection, whilst those in cluster 9 were only induced at 6hr post-infection. This may reflect differing functions of the various proteasome subunits.

3.2.3.7. A type I IFN-inducible cluster of genes in *Mtb*-infected macrophages

Strikingly, cluster 2 showed highly significant overlap with the canonical pathway "Interferon Signalling" following IPA analysis (Figure 3.4.1). This was of interest as type I IFN has been reported to play a negative role during *Mtb* infection (Manca *et al*, 2001; Manca *et al*, 2005; Ordway *et al*, 2007; Antonelli *et al*, 2010) and an interferon signature was recently reported in the blood of patients with active TB (Berry *et al*, 2010). Cluster 2 contained 257 transcripts, which were induced by *Mtb* at 6hr and 24hr post-infection (Figure 3.11). Genes in this cluster linked to interferon signalling by IPA included the transcription factors *Stat1*, *Stat2* and *Irf9*, the antiviral molecules *Mx1*, *Oas1a*, *Oas1g*, *Oas2* and *Oas3* and the IFN-inducible protein *Ifit3* (Figure 3.11). Several other known IFN-inducible genes were also present in cluster 2. These included the transcription factors *Irf2* and *Irf7* (Tamura *et al*, 2008); the IFN-inducible proteins *Ifi44* and *Ifit2*; and the antiviral gene *Isg20* (Espert *et al*, 2003) (Figure 3.11). In addition, cluster 2 included two members of the

tri-partite motif (TRIM) family of proteins, *Trim34* and *Pml* (TRIM19), which have recently been suggested to play an important role in regulating the innate immune response (McNab *et al*, 2010).

The IPA "interferon signalling" canonical pathway includes both type I IFN and IFNγ signalling. To determine the relative contribution to these pathways, transcripts in cluster 2 were overlaid onto the IPA "interferon signalling" pathway, with genes present in cluster 2 highlighted in red (Figure 3.12A). Genes involved in both type I IFN and IFNγ signalling were present in cluster 2. However, of the four genes involved in IFNγ signalling, three (*Stat1*, *Irf9* and *Psmb8*) were common to type I IFN signalling, explaining the induction of this pathway in the absence of IFNγ signalling, and illustrating the overlap between these two pathways (Figure 3.12A). The presence of genes unique to type I IFN signalling, *Stat2*, *Mx1*, *Oas* and *Ifit3* strongly suggested that type I IFN signalling was responsible for this cluster. In support of this, *Ifnb1*, the gene encoding IFNβ, was upregulated at 3hr post-infection (Figure 3.12B), in keeping with the appearance of type I IFN-inducible genes at 6hr post-infection (Figure 3.11). However, IFNβ protein was consistently undetectable from macrophages infected with *Mtb* by ELISA (data not shown), suggesting that low but biologically active concentrations of type I IFN are induced by *Mtb*. Taken together, these data strongly suggest that type I IFN is induced by macrophages, in response to *Mtb*, and that this feeds back to regulate gene expression. This is in agreement with previous studies reporting type I IFN induction in *Mtb*-infected macrophages (Stanley *et al*, 2007; Pandey *et al*, 2009).

3.2.3.8. Downregulated clusters are associated with DNA replication, metabolism and cell-division

Of the clusters that were downregulated following *Mtb* infection, several showed significant overlap with GO terms related to metabolism and cell-division. This was particularly strong for cluster 7 (Figure 3.4.2) and clusters 13, 16 and 17 (Figure 3.4.3), which were downregulated between 3hr and 24hr post-infection (Figure 3.13-3.16).

Genes in clusters 7 and 17 were significantly associated with metabolic processes, with highly significant overlap with the GO terms "cellular macromolecule metabolic process" and "cellular metabolic process" for cluster 7 (Figure 3.4.2), and "catalytic activity" and "metabolic process" for cluster 17 (Figure 3.4.3). This included numerous enzymes involved in metabolism and catabolic processes. Cluster 7 contained *B4galt1* (beta 1,4-galactosyl transferase), *Cbr1* (carbonyl reductase 1), *Coasy* (CoA synthase), *Dhdh* (dihydrodiol dehydrogenase), *Gpt2* (glutamic pyruvate transaminase), *Lcmt1* (leucine carboxyl methyltransferase) and *Umps* (urindine monophosphate synthetase) (Figure 3.13). Cluster 17 contained *Acp6* (acid phosphatase 6), *Galt* (galatcose-1-phosphate urididdyltransferase), *Gnpda1* (glucosamine-6-phosphate deaminase 1) and *Plcb2* (phospholipase C, beta 2) (Figure 3.14).

Cluster 13, which was strongly downregulated at 3hr, 6hr and 24hr (Figure 3.15), contained genes with a significant overlap with GO terms involving cell-division, including "mitosis", "nuclear division" and "M phase of mitotic cell cycle" (Figure 3.4.3). Genes in cluster 13 associated with these terms were members of the cyclin family of proteins, *Cnnm2* and *Cnnm3*, which control cell division through activation of the cyclin dependent kinases; the centromere proteins *Cenph*, *Cenpk*,

Cenpm and *Cenpp*; and the kinesin family members *Kif11*, *Kif13A*, *Kif15*, *Kif18A*, *Kif20b*, *Kif21b*, *Kif23*, *Kif2c* and *Kif3a* (Figure 3.15), which control chromosome segregation during mitosis (Mandelkow and Mandelkow, 2002).

In keeping with a downregulation of genes involved in cell-division, the genes in cluster 16 were strongly associated with DNA replication, a vital stage in the cell-cycle (Figure 3.4.3). Genes in cluster 16 were downregulated at 6hr and 24hr following *Mtb* infection, although the downregulation was relatively mild compared to clusters 7, 13 and 19 (Figure 3.16). These genes were upregulated in the uninfected controls at 24hr post-infection, suggesting that DNA replication may occur in uninfected macrophages (Figure 3.16). GO terms associated with cluster 16 included "DNA metabolic process" and "DNA replication (Figure 3.4.3). IPA confirmed this association, as cluster 16 was significantly linked with the IPA pathways "cell cycle control of chromosomal replication" and "pyrimidine metabolism" (Figure 3.4.3). The genes in cluster 16 that linked to DNA replication pathways included several members of the minichromosome maintenance complex, *Mcm3*, *Mcm5*, *Mcm6*, *Mcm7* and *Mcm10*, which is involved in the initiation and elongation stages of DNA replication (Figure 3.16). Also in cluster 16 are two subunits of the GINS complex, *Gins1* and *Gins2*, which are also involved in DNA replication (De Falco *et al*, 2007), and a subunit of the DNA polymerase enzyme, *Pole* (Figure 3.16).

In summary, analysis of clusters 7, 13, 16 and 17 strongly suggests that macrophages down regulate genes involved in metabolism, cell-division and DNA replication in response to *Mtb* infection. This may be to allow the macrophages to direct resources towards antimicrobial processes, such as the production of cytokines, chemokines and antimicrobial molecules.

3.2.3.9. The downregulated cluster 10 is associated with lysosome function

Although the majority of downregulated clusters were associated with either metabolism or cell-division, genes in cluster 10 showed a significant association with GO terms relating to the lysosome, including "lytic vacuole", "lysosome" and "vacuole" (Figure 3.4.2). This was intriguing as lysosomes and lysomomal enzymes are vital for protection against intracellular pathogens such as *Mtb*, as fusion of vacuoles containing *Mtb* with lysosomes leads to the destruction of the bacteria and the generation of peptides for antigen presentation (Pieters, 2008). The genes in cluster 10 associated with lysosomes were predominantly genes encoding lysosomal enzymes. This included peptidases such as the cathepsins, *Ctsf* and *Ctsb*; *Galc* (galactosylceramidase), *Hexa* (hexosaminidase) and *Tpp1* (tripeptidyl peptidase 1). In addition, several enzymes involved in carbohydrate digestion were present in cluster 10, including *Mann2b1* (mannosidase) and *Gusb* (glucoronidase B) (Figure 3.17). Given the importance of the lysosome in mediating *Mtb* killing in macrophages, it is possible that this downregulation of lysosomal genes is a virulence strategy of *Mtb*, designed to avoid macrophage killing and enabling *Mtb* to persist within the cell.

3.3. Discussion

The interactions between *Mtb* and macrophages are complex, with the macrophage attempting to eliminate the bacteria, and *Mtb* trying to subvert these processes (Pieters, 2008). The outcome of these interactions may be critical in determining the progression of the disease. Here we carried out transcriptional profiling of *Mtb*-infected macrophages over an extended time course. This revealed that the macrophage response to *Mtb* changes greatly over time, with genes induced and repressed with varying kinetics. We separated these genes into 24 clusters using *k*-means clustering, and analysis of these with IPA and GO analysis found associations with a wide range of important processes, including inflammation, antigen presentation, apoptosis, protein ubiquitination, sterol biosynthesis, cell-division and metabolism.

3.3.1. The early transcriptional response to *Mtb* infection

Previous microarray studies of *Mtb*-infected macrophages did not address the response of macrophages to *Mtb* earlier than 4hr post-infection (Ehrt *et al*, 2001; Chaussabel *et al*, 2003; Shi *et al*, 2003; Wang *et al*, 2003; Shi *et al*, 2005; Tailleux *et al*, 2008; Koo *et al*, 2012) (Table 3.1). We show here that although the majority of macrophage transcriptional changes occur at 3hr post-infection and later, a small number of transcripts were upregulated at between 30mins and 1hr post-infection. *k*-means clustering showed that these transcripts comprised two main clusters; one with sustained upregulation up to 24hr post-infection (cluster 8), associated with induction of cytokines, chemokines and NF-κB signalling, and a second with transient upregulation, containing transcripts which peaked at 1hr post-infection and returned to baseline by 3hr post-infection (cluster 15), associated with transcriptional

regulation and ERK MAP kinase signalling. This illustrates the importance of including early time points in this analysis, particularly with regard to the transcripts in cluster 15, which would not have been detected had the analysis started at 3hr post-infection.

Many of the genes in cluster 8 were pro-inflammatory in nature, such as the cytokines *Tnf* and *Il1b*, and the chemokines *Ccl2*, *Ccl3*, *Ccl4*, *Ccl7*, *Cxcl1* and *Cxcl2*. The production of chemokines in particular results in the influx of further immune cells into the site of infection, which is a vital process during *Mtb* infection as it culminates in the formation of the granuloma which sequesters *Mtb* within foci of immune cells (Flynn *et al*, 2011). For example, loss of *Ccl2* and *Ccl7* signalling in *Ccr2*$^{-/-}$ mice infected with *Mtb* led to a complete loss of monocyte recruitment to the lung, and failure to control the infection (Peters *et al*, 2001). TNFα and IL-1β are also highly pro-inflammatory in nature, although their precise function in the immune response to *Mtb* is unclear (Cooper *et al*, 2011). However, loss of either of these cytokines renders mice highly susceptible to *Mtb* infection (Flynn *et al*, 1995; Mayer-Barber *et al*, 2011). Thus, this early production of cytokines and chemokines by *Mtb*-infected macrophages in the lung is likely to represent a vital first stage in mounting a protective immune response.

The transiently induced genes in cluster 15 included many transcription factors and transcriptional regulators, such as *Fos*, *Egr1*, *Jun*, *Myc* and the histone H3K27 demethylase *Jmjd3*. These transcription factors are likely to induce the transcription of the genes upregulated at later time points, leading to the cascade of gene activation observed. Further work will be needed to address the precise function of these transcription factors in the immune response to *Mtb*, making use of either specific inhibitors or gene knockouts. In particular, the induction of the histone

demethylase *Jmjd3* is notable, in the light of recent studies showing that bacteria can stimulate chromatin modifications in host cells as a virulence mechanism, to modify gene expression (Hamon and Cossart, 2008). For example, the *Listeria monocytogenes* virulence factor LntA was recently shown to target the chromatin repressor BAHD1, which resulted in increased expression of type III IFN (Lebreton *et al*, 2011). To date, *Mtb* virulence has not been linked to chromatin remodelling, but the early induction of a histone demethylase in macrophages suggests that this may occur, and warrants further investigation.

3.3.2. A type I IFN signature in *Mtb* infected macrophages

Using IPA analysis, we identified a cluster of genes strongly linked to IFN signalling. This cluster (cluster 2) was induced at 6hr and 24hr post-infection, and contained many well known IFN-inducible genes including the transcription factors *Stat1*, *Stat2* and *Irf9* and the antiviral genes *Mx1*, *Oas1a*, *Oas1g*, *Oas2*, *Oas3* and *Isg20* (Stark *et al*, 1998; Sadler and Williams, 2008). Induction of this cluster occurred subsequently to the expression of IFNβ mRNA at 3hr post-infection, strongly suggesting that type I IFN was responsible for the induction of this cluster. Although IFNβ was not detectable at the protein level by ELISA, type I IFNs are known to often be produced at low but biologically active levels (Gough *et al*, 2012).

Several previous studies have reported induction of type I IFNs, particularly IFNβ, in macrophages infected with *Mtb* (Giacomini *et al*, 2001; Stanley *et al*, 2007; Leber *et al*, 2008; Pandey *et al*, 2009; Manzanillo *et al*, 2012). This was shown to depend on cytosolic recognition of *Mtb* or *Mtb*-components by either NOD2 (Leber *et al*, 2008; Pandey *et al*, 2009) or a cytosolic DNA sensor (Manzanillo *et al*, 2012). In addition, the upregulation of type I IFN inducible genes has been reported in

human macrophages and DCs infected with *Mtb* (Chaussabel *et al*, 2003; Tailleux *et al*, 2008; Wu *et al*, 2012). IFN-inducible genes are also upregulated in the blood of patients with active TB, but not those with latent TB or healthy controls (Berry *et al*, 2010). Induction of type I IFN and IFN-inducible genes seems to be common to both human and mouse cells infected with *Mtb*, and may play an important role during infection.

Mouse models have shown that type I IFN plays a detrimental role in the immune response to *Mtb*, with mice deficient in type I IFN signalling having lower bacterial loads following infection (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007). The induction of type I IFN may therefore be a virulence strategy of *Mtb* to subvert the macrophage response. This is supported by the fact that induction of type I IFN by *Mtb*, both *in vitro* and *in vivo*, depends on the ESX-1 secretion system (Stanley *et al*, 2007). This secretion system is crucial for *Mtb* virulence, and loss of the genes encoding it accounts for the attenuation of BCG (Pym *et al*, 2002).

The observation of type I IFN-inducible genes in *Mtb*-infected macrophages prompted us to further investigate how type I IFN regulates the macrophage response, and the results are shown in Chapter 4.

3.3.3. *Mtb* induction of genes involved in cholesterol biosynthesis

We found that a number of genes involved in the biosynthesis of steroids, including cholesterol, were upregulated following *Mtb* infection (cluster 9; Figure 3.4.2). This was confirmed by both GO and IPA analysis and included the enzymes phosphomevalonate kinase (*Pmvk*) and mevalonate decarboxylate (*Mvd*), which are involved in the mevalonate pathway, a biosynthetic pathway that produces the precursors for a wide range of steroids, including cholesterol (Goldstein and Brown,

1990). These results are in agreement with a recent study by Koo *et al* (2012), which described the induction of steroid biosynthesis genes, including *Mvd*, by the hypervirulent strain of *Mtb* HN878, but not by the less virulent strain CDC1551. We show here that H37Rv also induces genes involved in steroid biosynthesis. Induction of these genes by *Mtb* therefore appears to be strain specific, and induction of these genes to differing levels may in part explain differences in virulence observed between different strains of *Mtb*.

The induction of steroid biosynthesis genes may represent a virulence strategy of *Mtb*. It was reported that *Mtb* is able to metabolise cholesterol, and this is dependent upon genes in the *mce4* operon, which encodes for transporters that transport cholesterol across the bacterial cell wall (Pandey *et al*, 2009). The importance of this was shown by the fact that mutant *Mtb* deficient in *mce4* were unable to survive in the lung during the chronic stages of infection in mice, showing that cholesterol metabolism is required for *Mtb* persistence *in vivo* (Pandey *et al*, 2009). In human TB the production of lipids such as cholesterol leads to the appearance of "foamy" macrophages in the lung, characterised by intracellular lipid droplets (Russell *et al*, 2009). Electron microscopy has shown that *Mtb* is closely associated with these lipid droplets in the lungs of TB patients, and it has been hypothesised that this provides an energy source which enables *Mtb* to persist within the lungs during the latent stage of infection (Pieters, 2001; Russell *et al*, 2009). In addition, cholesterol is required for the entry of mycobacteria into macrophages, through a variety of receptors (Gatfield and Pieters, 2000; Peyron *et al*, 2008). Our results therefore suggest that *Mtb* generates the production of these steroids such as cholesterol by manipulating host metabolism, through inducing enzymes involved in

the generation of lipids, and that this may enable *Mtb* to enter macrophages, and persist within them.

3.3.4. Induction of apoptosis related genes by *Mtb*

A prominent cluster of genes upregulated at 3hr, 6hr and 24hr post-infection, cluster 19 (Figure 3.4.4), was strongly associated with GO terms relating to the death of cells, including "apoptosis" and "programmed cell-death". This strongly suggested that macrophages were undergoing cell death in response to *Mtb*. There are two main mechanisms leading to cell death; apoptosis and necrosis (Behar *et al*, 2011). Necrosis is characterised by the disruption of the plasma membrane, whereas during apoptosis the integrity of the plasma membrane is preserved, and the cell breaks up into many small vesicles (blebbing) (Behar *et al*, 2011). The death of macrophages by apoptosis is known to promote the host response to *Mtb*, first because it leads to reduced viability of *Mtb* (Oddo *et al*, 1998; Divangahi *et al*, 2009) and second because the apoptotic vesicles contain *Mtb* antigens, which are taken up by DCs and used to cross-prime $CD8^+$ T cells, leading to an enhanced $CD8^+$ T cell response (Schaible *et al*, 2003; Winau *et al*, 2006; Divangahi *et al*, 2009). The expression of pro-apoptotic genes, such as *Fas* and *Bcl2l11*, is therefore likely to be protective in the host response. In support of this, apoptosis mediated through the *Fas* receptor has been shown to reduce *Mtb* viability in human macrophages (Oddo *et al*, 1998).

However, *Mtb* has also been shown to actively inhibit apoptosis in macrophages, leading instead to necrosis which promotes inflammation and bacterial dissemination (Behar *et al*, 2011). Evidence for this comes from studies showing that virulent strains of *Mtb* induce greater levels of necrosis compared to avirulent

strains, which are more prone to induce apoptosis (Chen *et al*, 2006; Gan *et al*, 2008; Divangahi *et al*, 2009). The virulent strain of *Mtb*, H37Rv, has consistently been shown to induce greater levels of necrosis in macrophages compared to the avirulent strain H37Ra, through increased disruption of the mitochondrial membrane (Chen *et al*, 2006) and manipulation of the synthesis of eicosanoids (Gan *et al*, 2008; Divangahi *et al*, 2009). We also observe the upregulation of anti-apoptotic genes in *Mtb*-infected macrophages, specifically two members of the inhibitor of apoptosis (IAP) family, *Birc2* and *Birc3*, which prevent apoptosis by inhibiting the activity of caspases (Siegel, 2006). *Mtb* may induce expression of *Birc2* and *Birc3* as a virulence mechanism, in order to prevent apoptosis, resulting in macrophage death by necrosis and increased bacterial dissemination.

3.3.5. Upregulation of MHC class I-related antigen processing genes in *Mtb*-infected macrophages

Cluster 4 (Figure 3.4.1) contained many genes involved with processing of antigens for presentation on MHC class I molecules. Antigens presented on MHC are derived from the cytosol of cells, and typically derive from cytosolic pathogens such as viruses (Vyas *et al*, 2008). Peptide antigens presented on MHC class I molecules are then recognised by $CD8^+$ T cells, and this leads to death of the infected cell (Vyas *et al*, 2008). The generation of these peptides occurs through the action of the proteasome, which degrades proteins into short peptides which are then transported into the ER by the TAP (transporter associated with antigen processing) transporter. In the ER, peptides are further processed by peptidases such as the ER aminopeptidase (encoded by *Erap1*), loaded onto MHC class I molecules and transported to the cell surface (Vyas *et al*, 2008). Cluster 4 contained a large number

genes encoding subunits of the proteasome, two MHC class I molecules, H2-K1 and H2-M2, and the aminopeptidase *Erap1*. This strongly suggested that the MHC class I antigen presentation pathway was activated in *Mtb*-infected macrophages.

$CD8^+$ T cells are known to recognise *Mtb* antigens during infection, and although not as important as $CD4^+$ T cells, still play an important role in containing bacterial growth (North and Jung, 2004). However, given that *Mtb* resides in the phagosome, which is restricted to the MHC class II pathway, how *Mtb* antigens enter the MHC class I pathway and activate $CD8^+$ T cells has been an area of much research. Two pathways have been proposed for the entry of exogenous antigens into the MHC class I pathway (a process known as cross-priming or cross-presentation) (Vyas *et al*, 2008). First, antigens may exit from the phagosome into the cytosol, be processed by the proteasome and enter the ER via the TAP transporter, as in classical MHC class I antigen processing (the cytosolic pathway) (Vyas *et al*, 2008). Second, antigens in the phagosome may traffic to a vesicle containing recycled MHC class I molecules, and thus bypass the proteasome and ER (the vesicular pathway) (Vyas *et al*, 2008). In the context of *Mtb*, several studies have shown that cross-presentation of a variety of *Mtb* antigens by human monocyte derived DCs requires the proteasome and the TAP transporter, suggesting that *Mtb* antigens enter the cytosol, although exactly how *Mtb* antigens exit from the phagosome remains unclear (Lewinsohn *et al*, 2006; Grotzke *et al*, 2009; Grotzke *et al*, 2010). However, in contrast to these studies, cross-presentation of the 19kDa antigen of *Mtb* was shown to be TAP-independent (Neyrolles *et al*, 2001) and cross-priming of apoptotic vesicles by bystander-DCs was also shown to be proteasome-independent (Schaible *et al*, 2003), supporting a role for the vesicular pathway. Our results support a role for the cytosolic pathway, as many genes involved in this pathway, particularly

proteasome subunits and the TAP transporter, were upregulated by macrophages in response to *Mtb*. However, this does not exclude a role for the vesicular pathway, and it may be that both pathways are important depending on the *Mtb* antigen.

3.3.6. Macrophages downregulate many genes in response to *Mtb* infection

It was notable that, despite infection with *Mtb* being regarded as an "activating" signal for macrophages, almost as many transcripts were downregulated in response to infection, as were upregulated. This effect has been consistently observed in microarray studies of innate immune cells treated with a wide variety of pathogens and pathogen derived products including *Mtb* (Nau *et al*, 2002; Chaussabel *et al*, 2003; Ramsey *et al*, 2008), but the function of these genes has not been investigated in detail. In our study, GO analysis of several clusters of downregulated transcripts found them to be significantly associated with metabolism, cell-division and DNA replication. It therefore seems likely that macrophages shut off these processes in order to direct resources towards the antimicrobial response, such as the production of cytokines, chemokines and antimicrobial molecules as shown above.

However, one cluster of downregulated genes was associated with lysosome function. Lysosomes are intracellular vesicles of low pH which contain various hydrolytic enzymes capable of destroying phagocytosed material, and the downregulated genes in this cluster included several lysosomal enzymes such as Cathepsin F (*Ctsf*) and Cathepsin B (*Cfsb*) (Vergne *et al*, 2004). Normally, phagosomes containing bacteria fuse with lysosomes, leading to bacterial killing and the generation of antigens for presentation on MHC class II molecules (Vyas *et al*,

2008). However, *Mtb* has evolved to block the maturation of the phagosome and its subsequent fusion with lysosomes, creating a "mycobacterial phagosome" in which it can replicate (Vergne *et al*, 2004). *Mtb* achieves this by preventing the accumulation of phosphatidylinositol 3-phosphate (PI3P) on the phagosome surface, a process which is required for phagosome maturation (Pieters, 2008). It is tempting to speculate that the downregulation of lysosome associated genes may represent an additional mechanism by which *Mtb* blocks phagosome-lysosome fusion. However, these genes are downregulated only at 24hr post-infection, well after the first entry of *Mtb* into the macrophages, and thus may instead be a natural response of macrophages to the establishment of infection. Further research will be needed to determine whether this represents a true virulence strategy of *Mtb*.

3.3.7. Use of IPA and GO analysis to investigate functions associated with differentially expressed genes

We used two independent strategies to try and determine the functions of clusters of genes generated by *k*-means clustering using GeneSpring. The first was gene ontology (GO) analysis, which uses the GO database, a resource where genes are annotated with terms relating to their function, based on the published literature (Harris *et al*, 2004). An example would be the cytokine TNFα, which is associated with the GO terms "cytokine" and "cytokine signalling" among others. A second approach, Ingenuity Pathway Analysis (IPA) was also used. We used IPA analysis to determine significant associations with canonical signalling pathways in IPA, which are built based on interactions reported in the literature (Calvano *et al*, 2005). STAT1, for example, would be linked to the IFN signalling pathway.

Our results highlight the pros and cons of both approaches. In general, IPA generated more specific information about the potential functions of different clusters. For example, IPA linked cluster 2 with IFN signalling, due to the presence of *Stat1*, *Stat2*, *Irf9* and *Mx1*. In contrast, GO analysis found associations with the "immune process" and "immune system process", vague terms which provide little information on the signalling pathways regulating these genes. However, GO often gave useful biological information on clusters in which no significant IPA clusters were found. For example, the downregulated clusters of genes linked to cell-division and metabolism by GO analysis showed no association with IPA pathways. This may reflect the fact that analysis of signalling pathways by IPA cannot encompass broad cellular functions such as metabolism.

In general, however, the results of both IPA and GO analysis should be treated with caution, as the information in these databases is generated from vast amounts of published literature, which may not always be scientifically validated. For example, an interaction or function of a gene reported in a cell-line may not reflect its true function in a primary cell or *in vivo*. Thus, it is important to validate suggested IPA and GO functions by manually searching the published literature to confirm these associations.

3.4. Figures

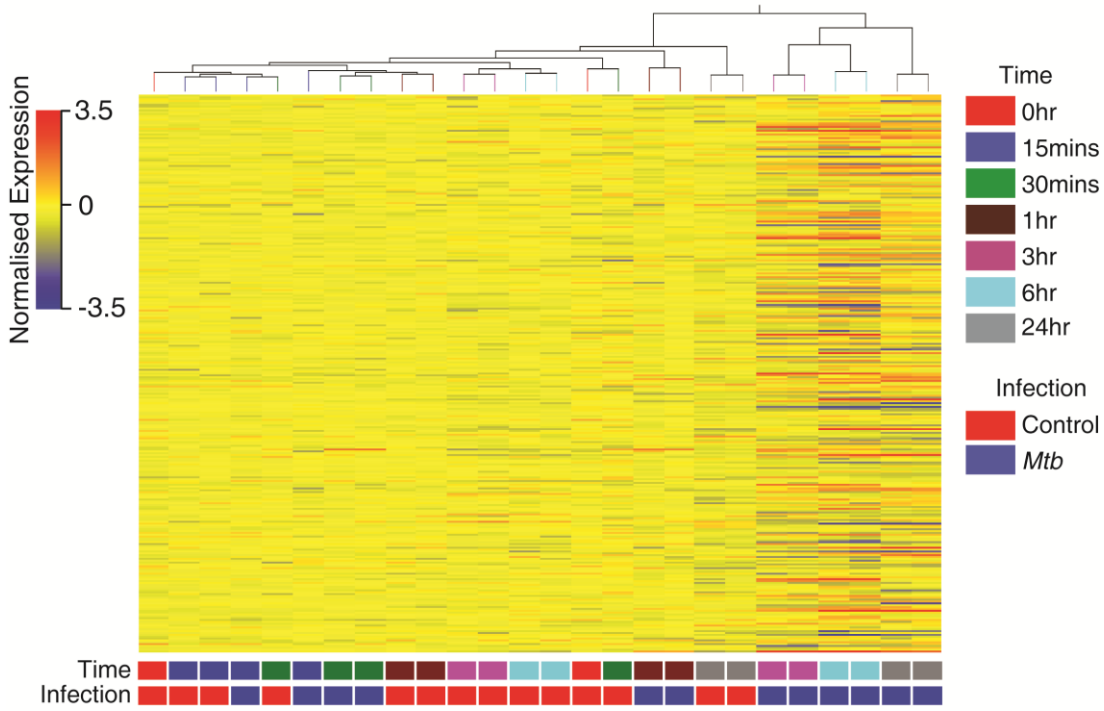

Figure 3.1. *Mtb*-infected samples at 3hr, 6hr and 24hr post-infection cluster separately from uninfected controls. WT macrophages were infected with *Mtb* and at the indicated time points post-infection RNA was harvested and analysed by microarray. Data was normalised, and undetectable transcripts were removed, as described in the Materials and Methods. Unsupervised clustering was then applied to the samples, using a Euclidian distance metric.

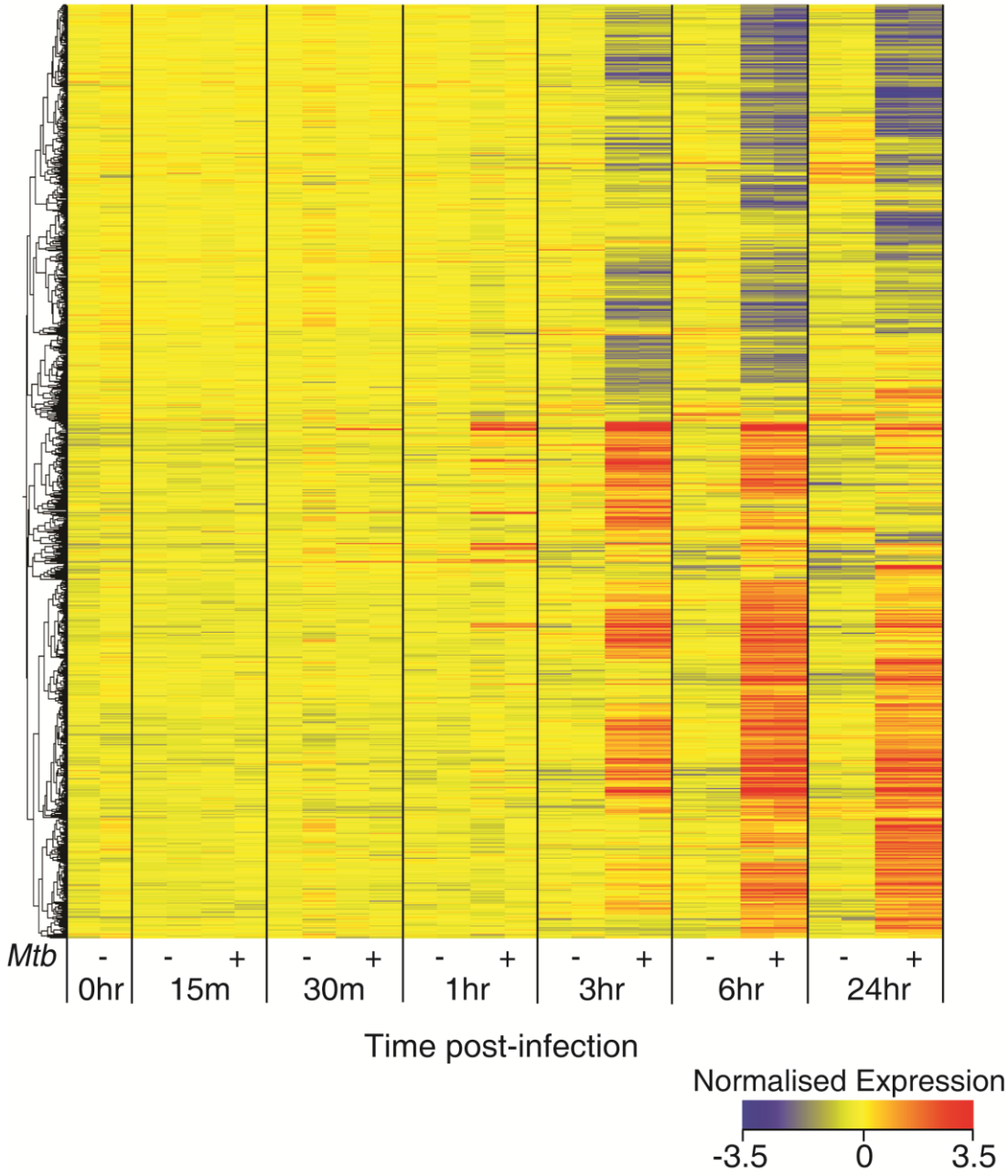

Figure 3.2. Macrophages infected with *Mtb* undergo dramatic transcriptional changes. WT macrophages were infected with *Mtb* and at the indicated time points post-infection RNA was harvested and microarray analysis was carried out. Data was normalised and undetectable transcripts were removed as described in the Materials and Methods. Differentially regulated transcripts were obtained by taking those that were at least 2-fold up- or downregulated in infected samples vs. controls at any time point, and those that were significantly different by two-way ANOVA $p<0.05$ with Benjamini Hochberg FDR multiple testing correction. This left 6479 transcripts, which were subjected to hierarchical clustering using Pearson centred distance metric and complete linkage. Normalised expression was visualised using a heat map.

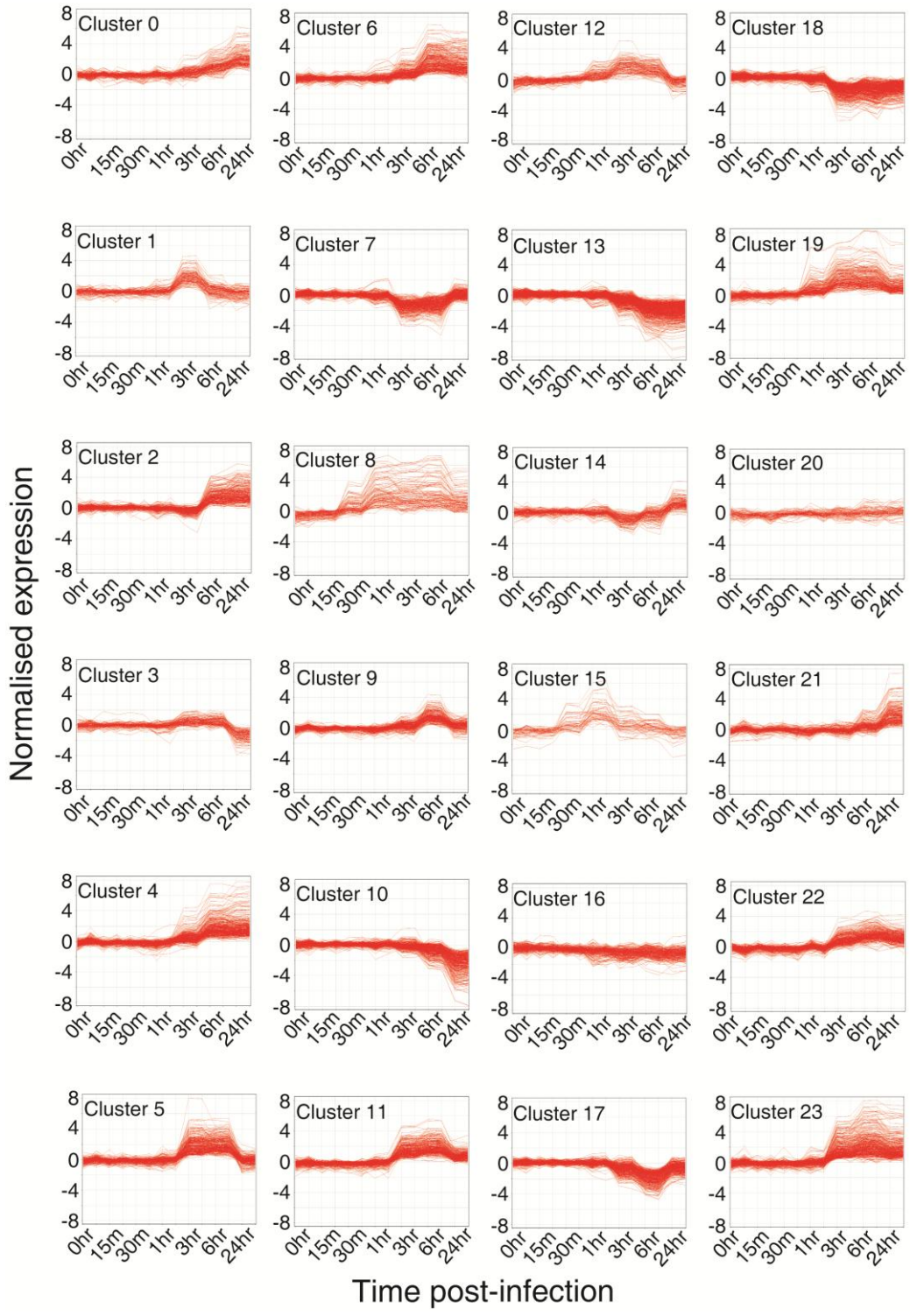

Figure 3.3. *Mtb* induced transcriptional changes can be separated into distinct clusters. The 6479 differently expressed transcripts in Figure 3.2 were separated into 24 clusters by *k*-means clustering, using a Pearson Centred distance metric. The normalised expression profile of the transcripts in each cluster is shown.

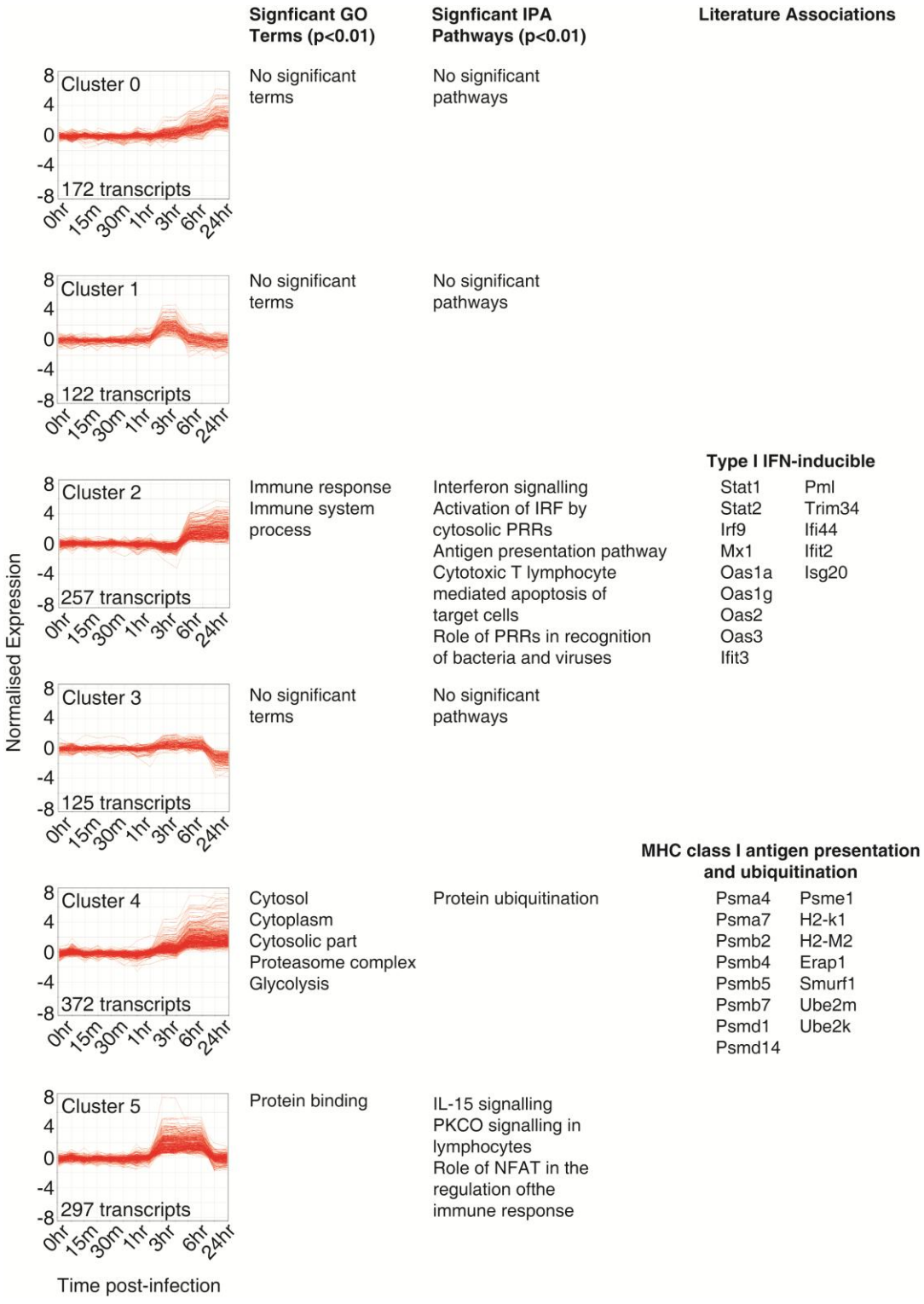

Figure 3.4.1. *Mtb*-induced transcriptional clusters are associated with a wide range of GO terms and IPA pathways. Each of the 24 *k*-means clusters described in Figure 3.3. were analysed by GO and IPA. GO terms and IPA pathways with significant overlap ($p<0.01$) are displayed next to expression profiles of the corresponding cluster. Associations of the clusters with functions and signalling pathways as determined by literature searching, and the genes associated, are also shown.

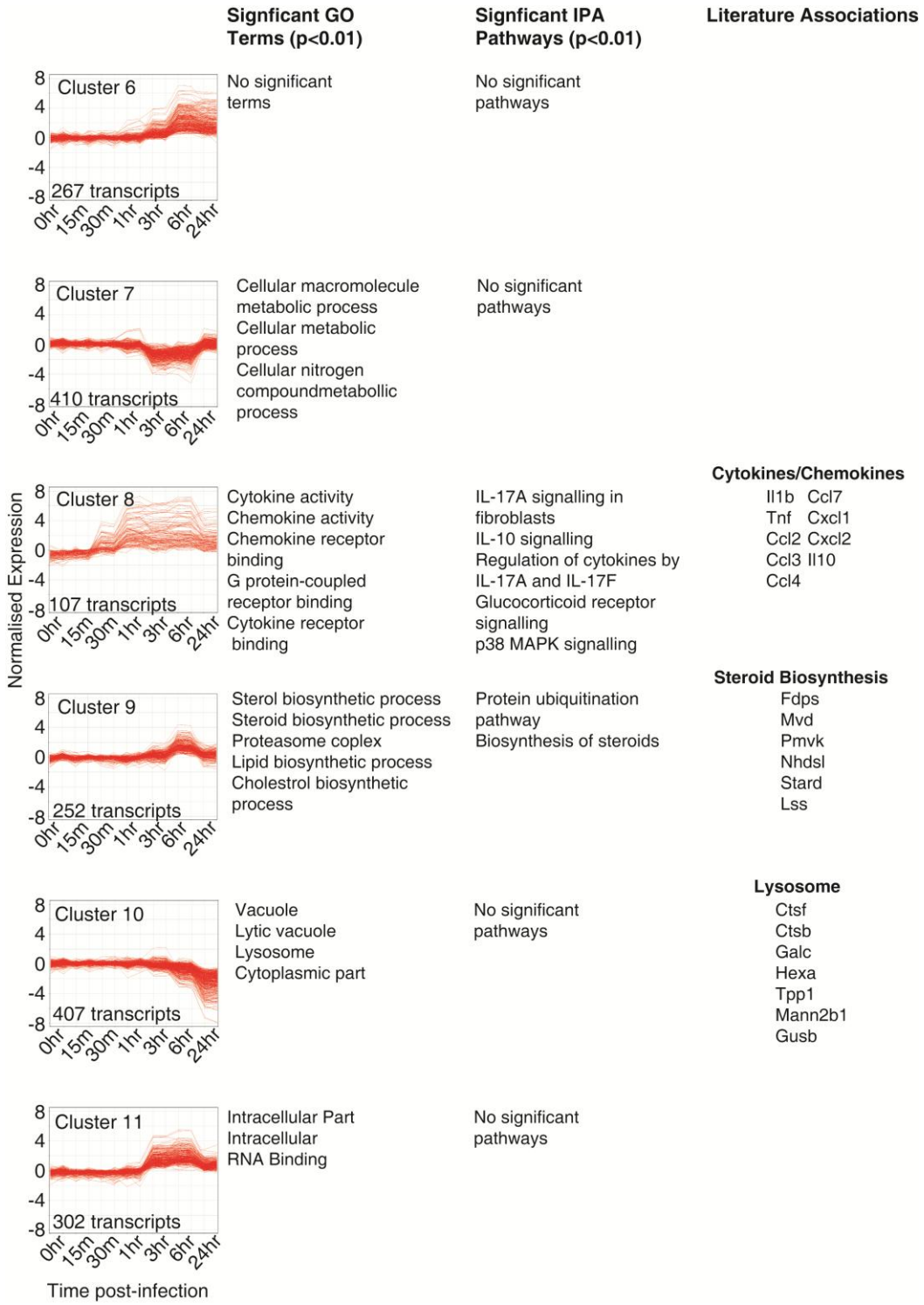

Figure 3.4.2. *Mtb*-induced transcriptional clusters are associated with a wide range of GO terms and IPA pathways. Each of the 24 *k*-means clusters described in Figure 3.3. were analysed by GO and IPA. GO terms and IPA pathways with significant overlap ($p<0.01$) are displayed next to expression profiles of the corresponding cluster. Associations of the clusters with functions and signalling pathways as determined by literature searching, and the genes associated, are also shown.

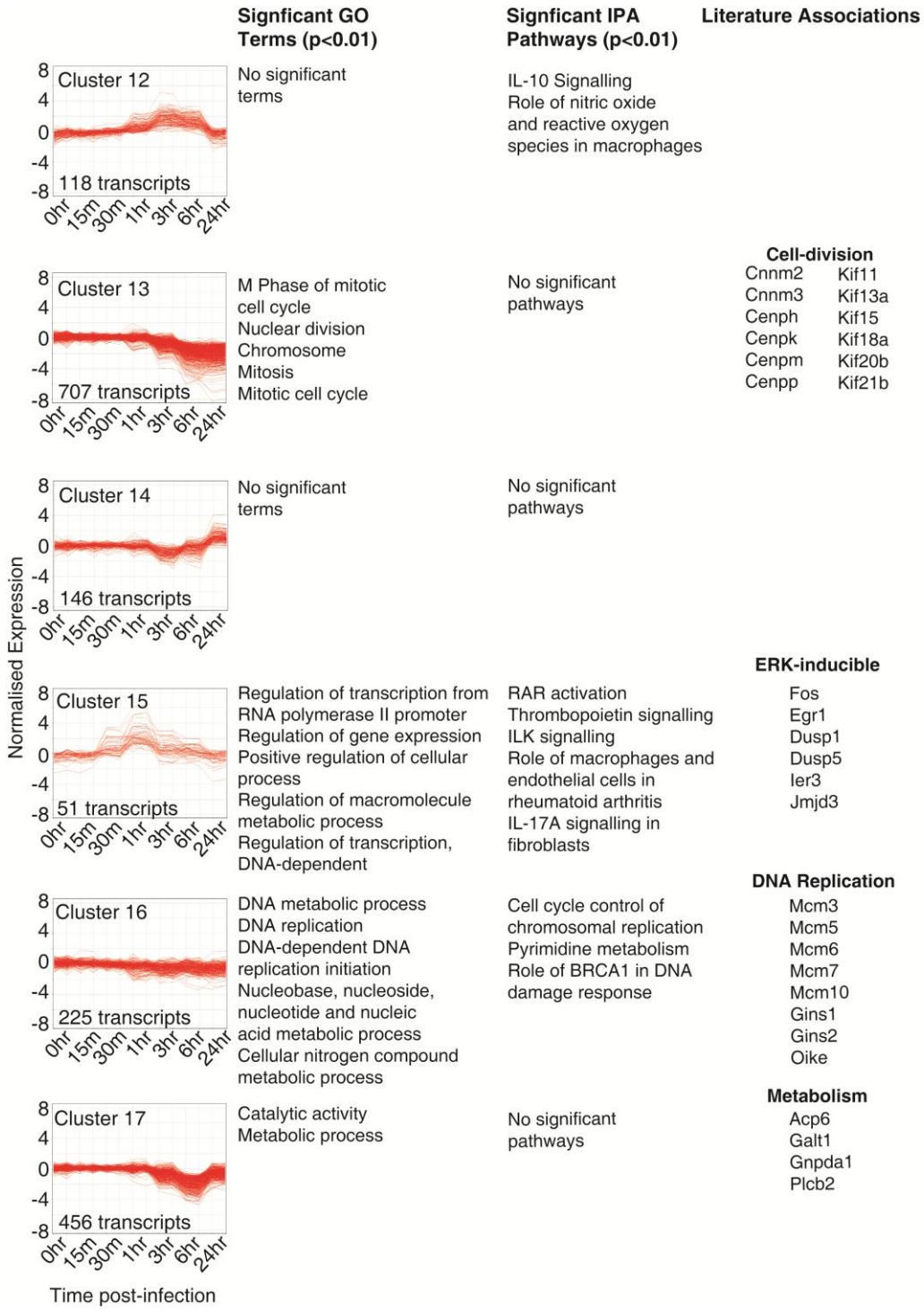

Figure 3.4.3. *Mtb*-induced transcriptional clusters are associated with a wide range of GO terms and IPA pathways. Each of the 24 *k*-means clusters described in Figure 3.3. were analysed by GO and IPA. GO terms and IPA pathways with significant overlap ($p<0.01$) are displayed next to expression profiles of the corresponding cluster. Associations of the clusters with functions and signalling pathways as determined by literature searching, and the genes associated, are also shown.

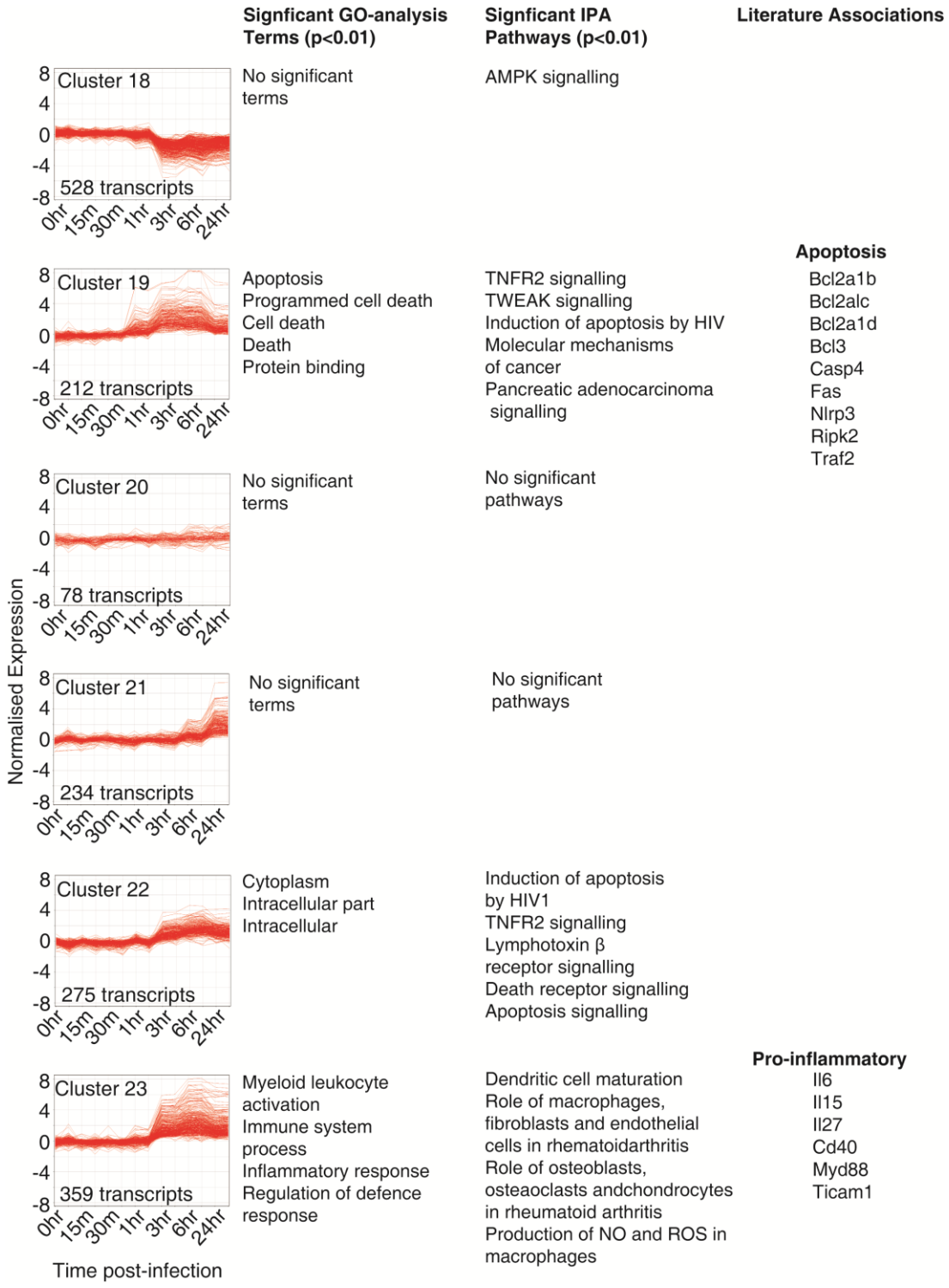

Figure 3.4.4. *Mtb*-induced transcriptional clusters are associated with a wide range of GO terms and IPA pathways. Each of the 24 *k*-means clusters described in Figure 3.3. were analysed by GO and IPA. GO terms and IPA pathways with significant overlap ($p<0.01$) are displayed next to expression profiles of the corresponding cluster. Associations of the clusters with functions and signalling pathways as determined by literature searching, and the genes associated, are also shown.

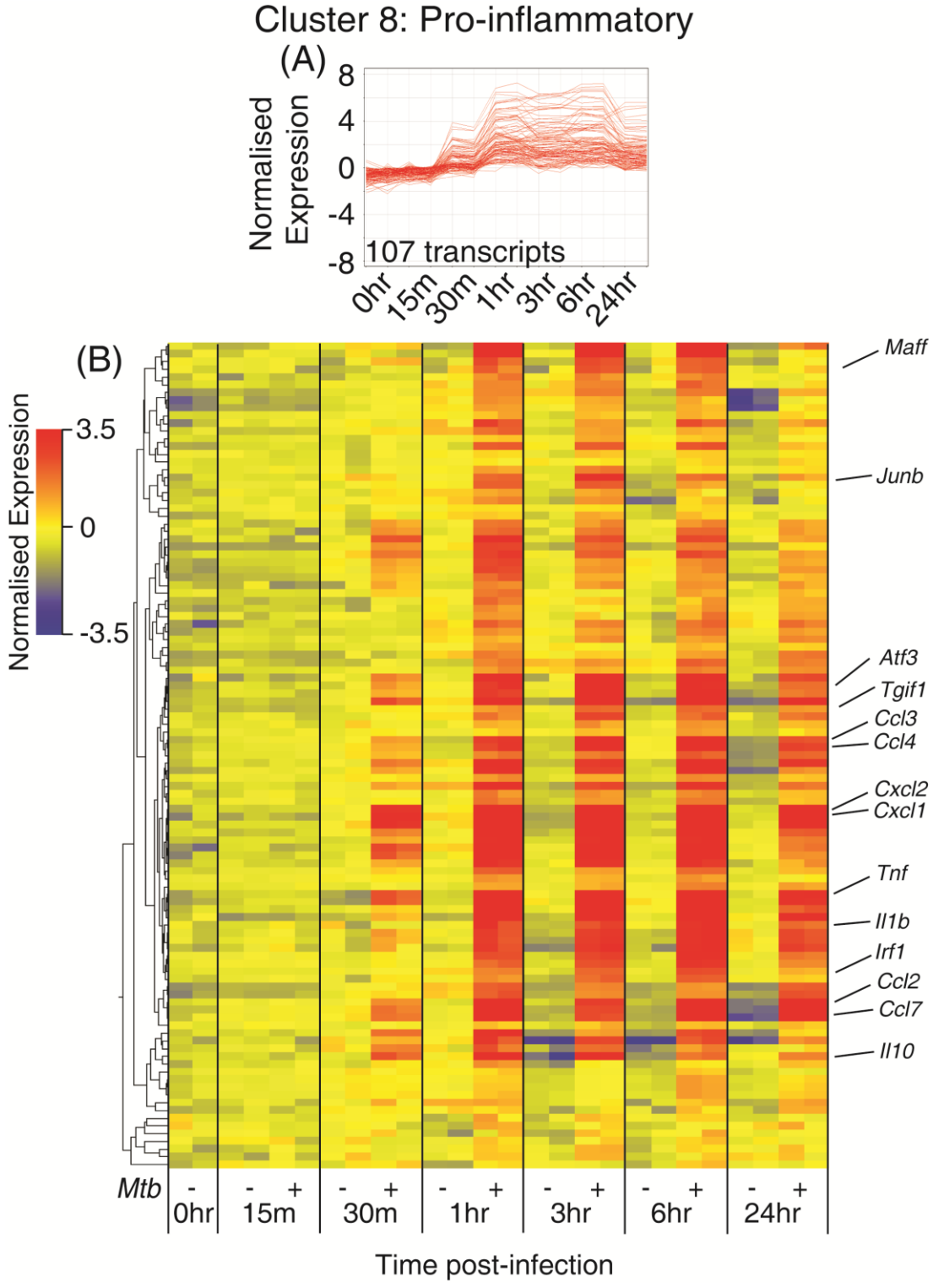

Figure 3.5. Cluster 8 is associated with pro-inflammatory functions. (A) Expression profile of transcripts cluster 8. (B) Transcripts in cluster 8 were clustered hierarchically using Pearson centred distance metric with complete linkage.

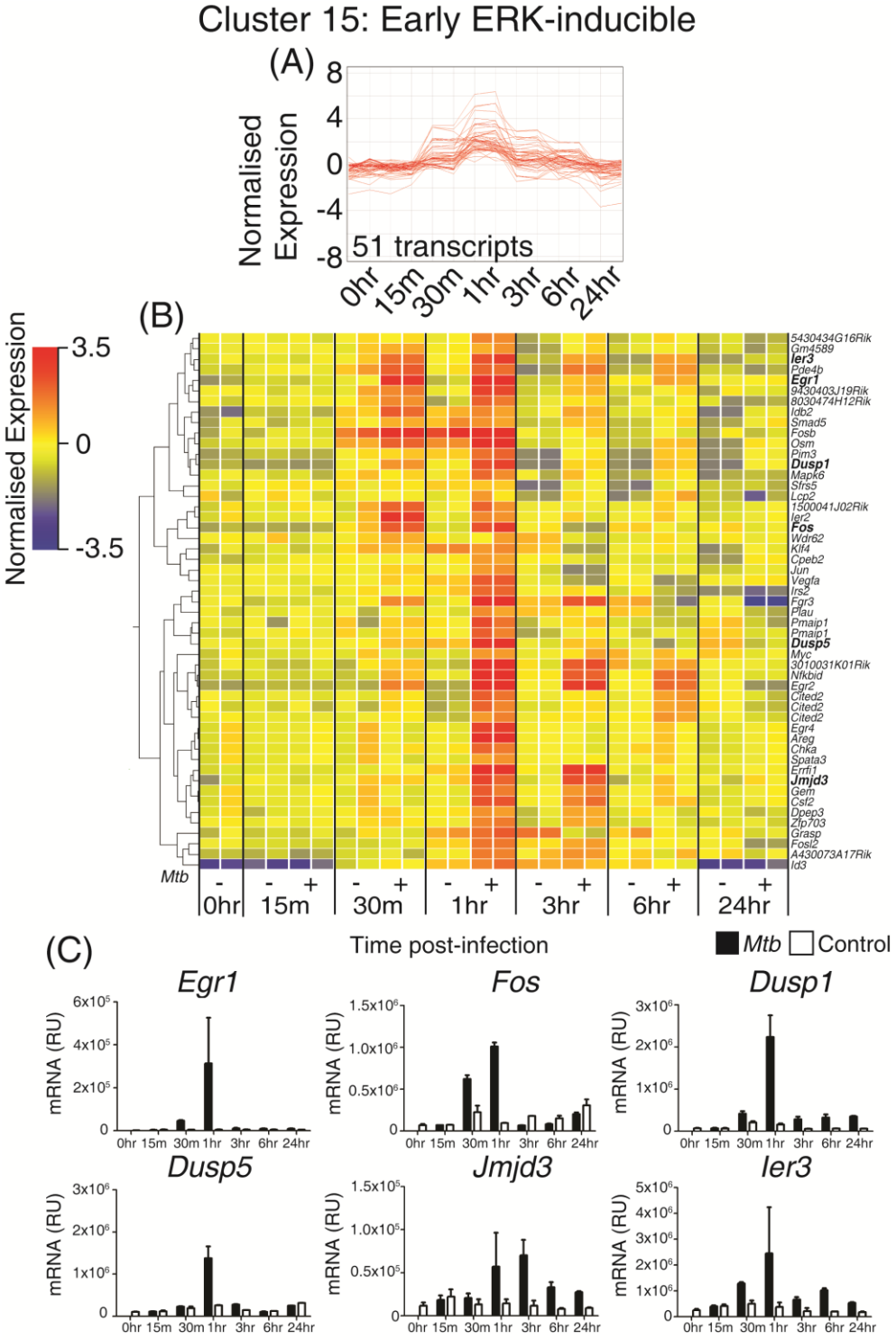

Figure 3.6. Cluster 15 is associated with ERK MAP kinase signalling. (A) Expression profile of cluster 15. (B) Genes in cluster 15 were clustered hierarchically using Pearson centred distance metric with complete linkage. (C) The same RNA was analysed by qPCR for expression of selected genes in cluster 15.

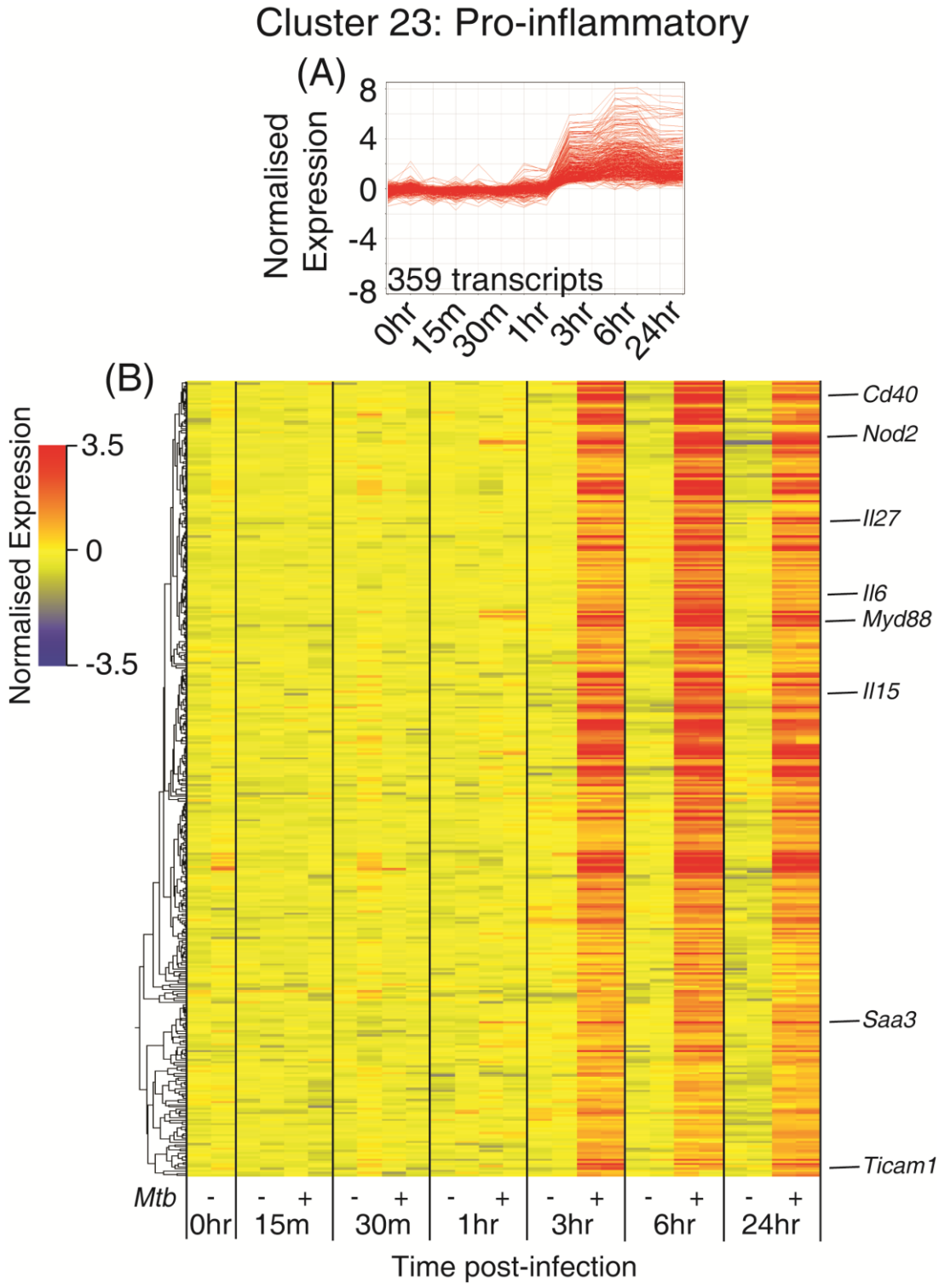

Figure 3.7. Cluster 23 is associated with pro-inflammatory functions. (A) Expression profile of cluster 23. (B) Transcripts in cluster 23 were clustered hierarchically using Pearson centred distance metric with complete linkage.

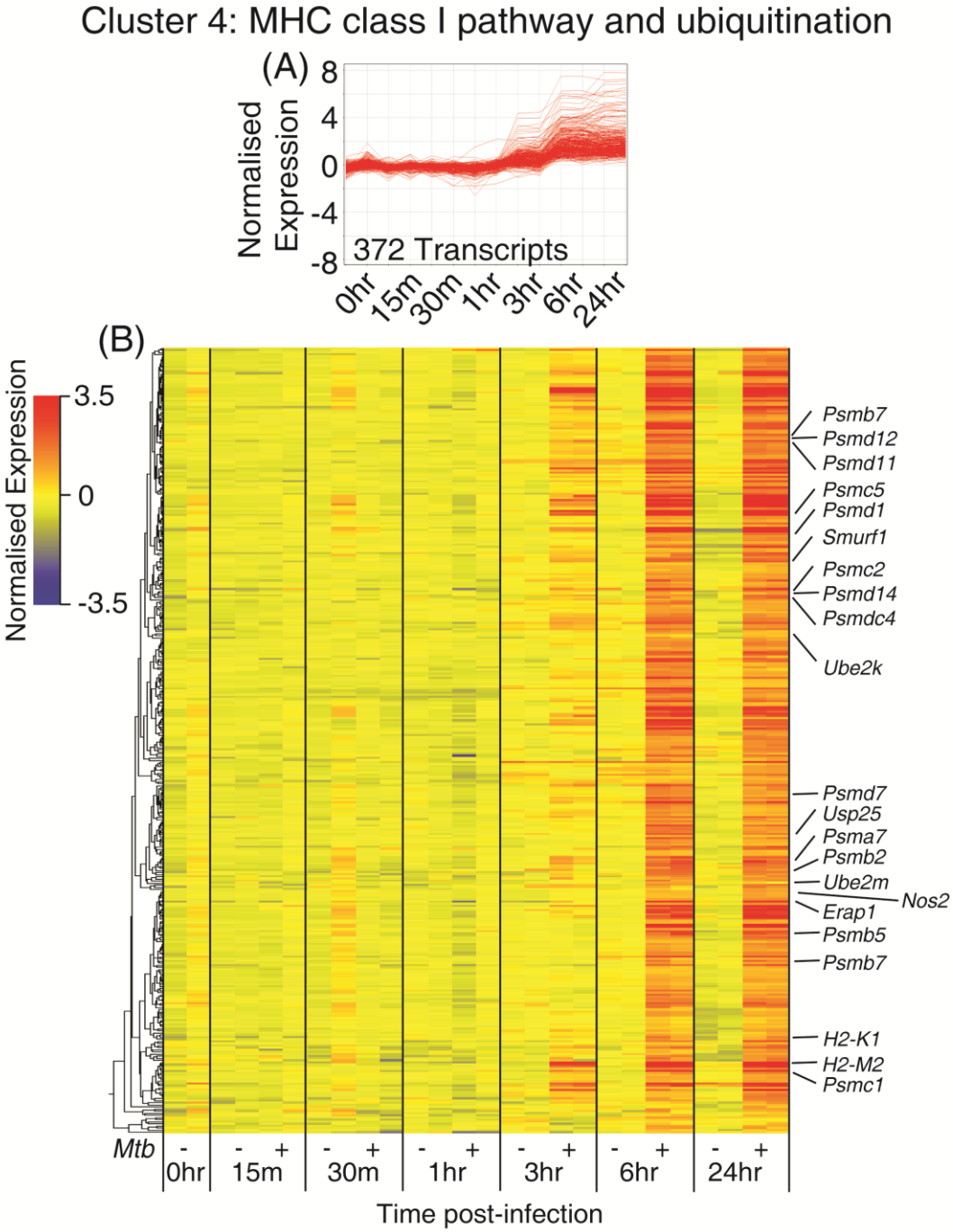

Figure 3.8. Cluster 4 is associated with the MHC class I antigen presentation pathway, and ubiquitination. (A) Expression profile of cluster 4. (B) Transcripts in cluster 4 were clustered hierarchically using Pearson centred distance metric with complete linkage.

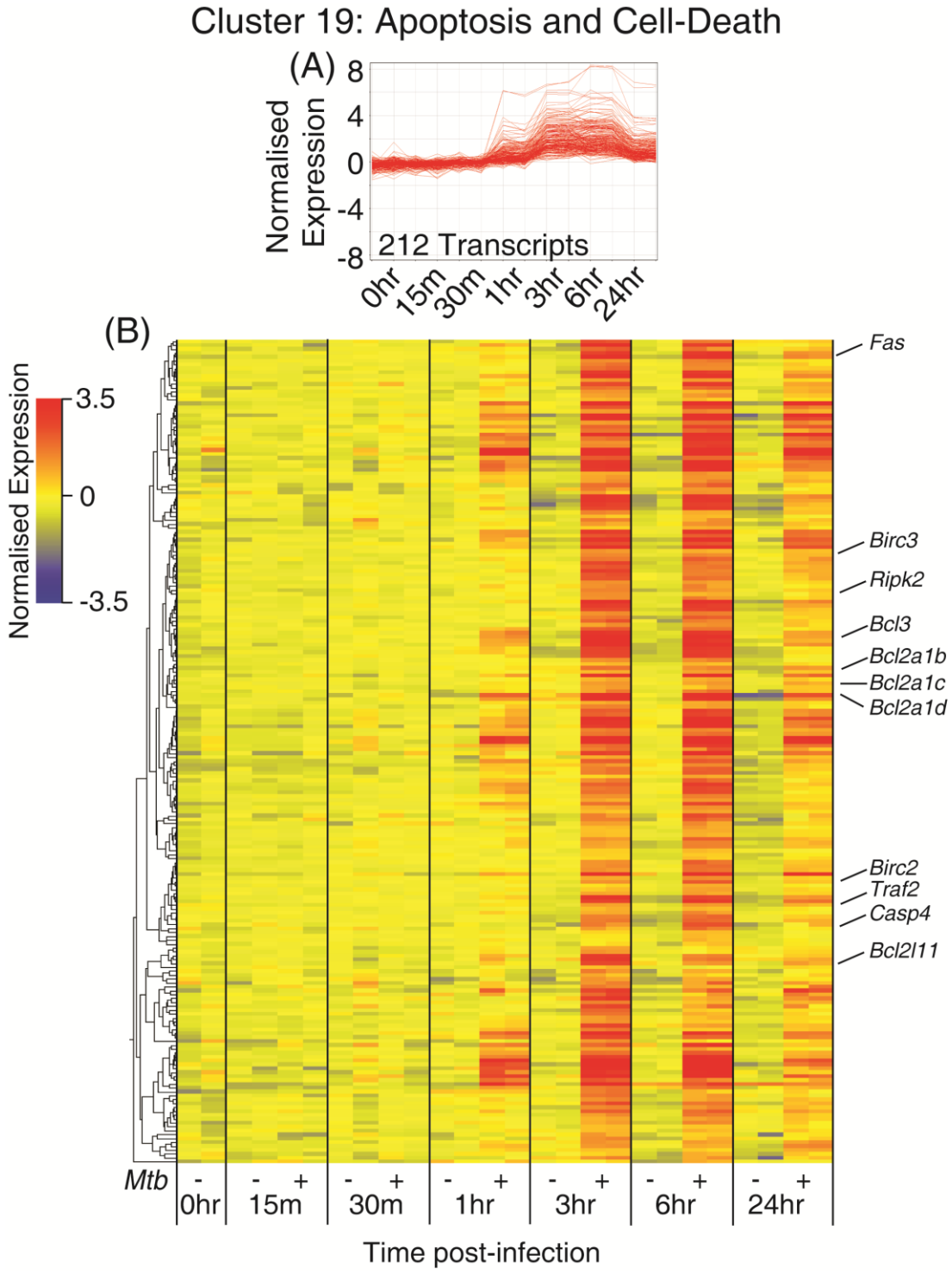

Figure 3.9. Cluster 19 is associated with apoptosis and cell-death. (A) Expression profile of cluster 19. (B) Transcripts in cluster 19 were clustered hierarchically using Pearson centred distance metric with complete linkage.

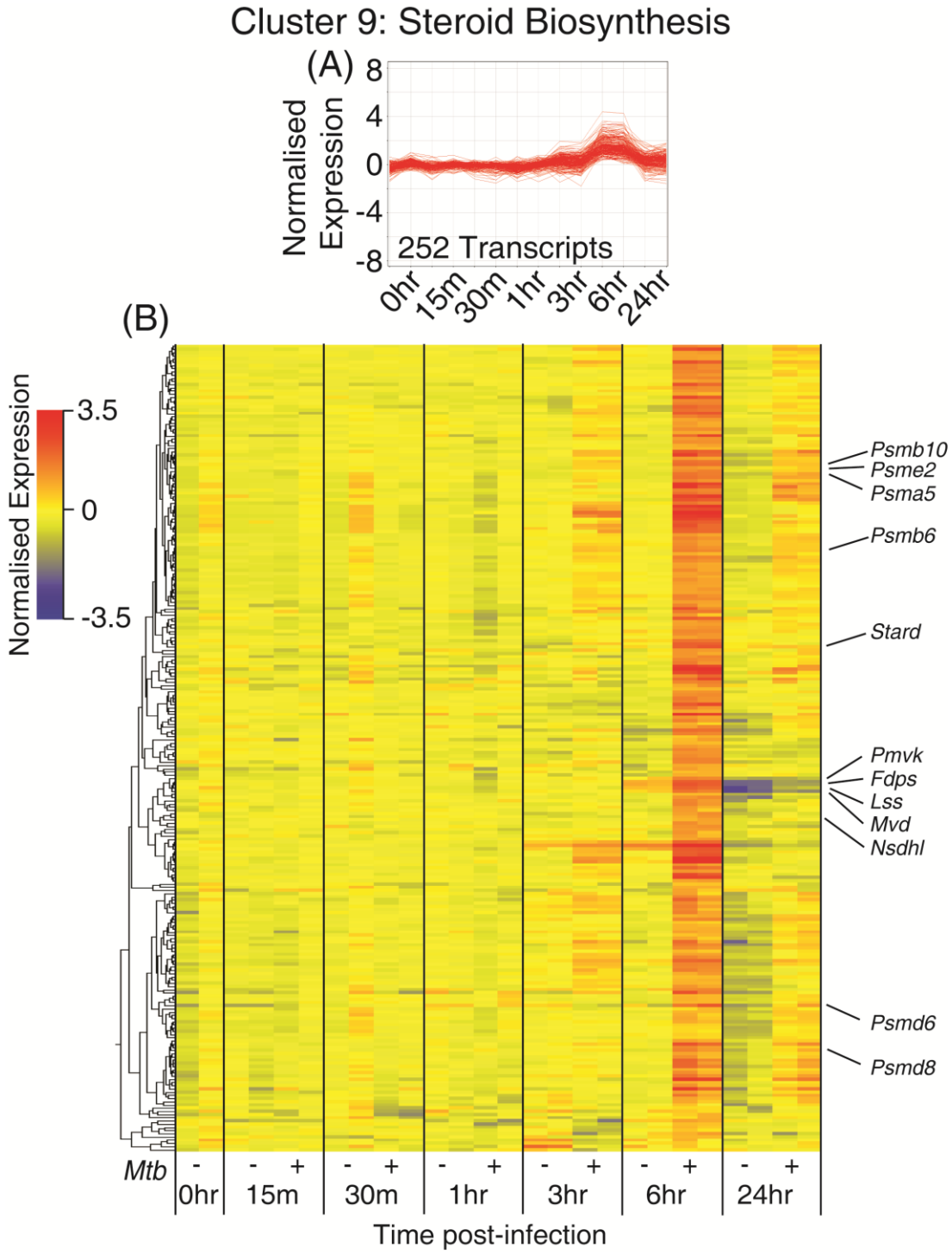

Figure 3.10. Cluster 9 is associated with steroid biosynthesis. (A) Expression profile of cluster 9. (B) Transcripts in cluster 9 were clustered hierarchically using Pearson centred distance metric with complete linkage.

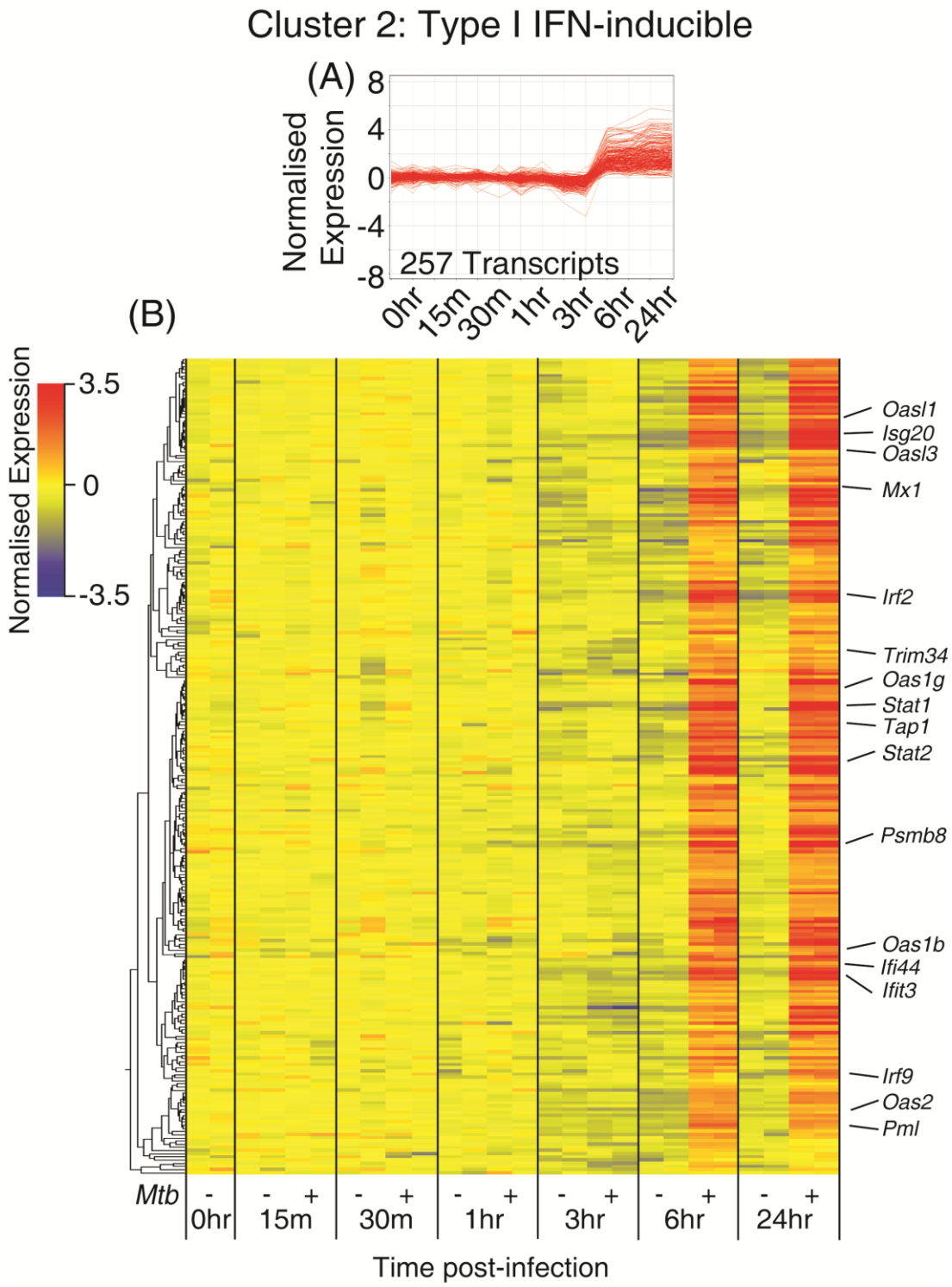

Figure 3.11. Cluster 2 is associated with IFN signalling. (A) Expression profile of cluster 2. (B) Transcripts in cluster 2 were clustered hierarchically using Pearson centred distance metric with complete linkage.

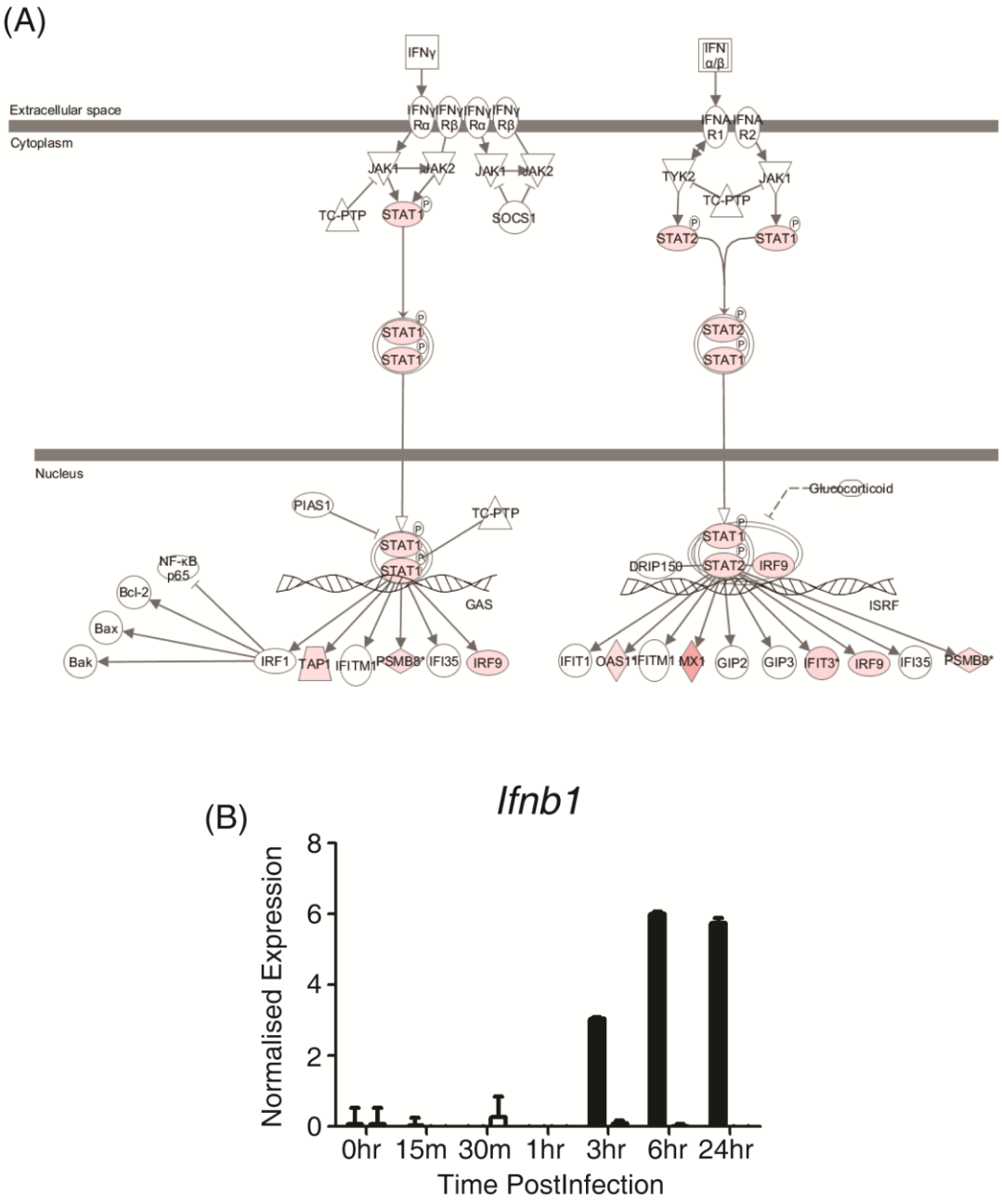

Figure 3.12. Type I IFN is strongly associated with genes in cluster 2. (A) Transcripts in cluster 22 were overlaid on the canonical pathway of IPA for Interferon Signalling. Genes over-represented in Cluster 2 are shaded in red. (B) Normalised Expression of *Ifnb1* determined by microarray analysis following *Mtb* infection of macrophages.

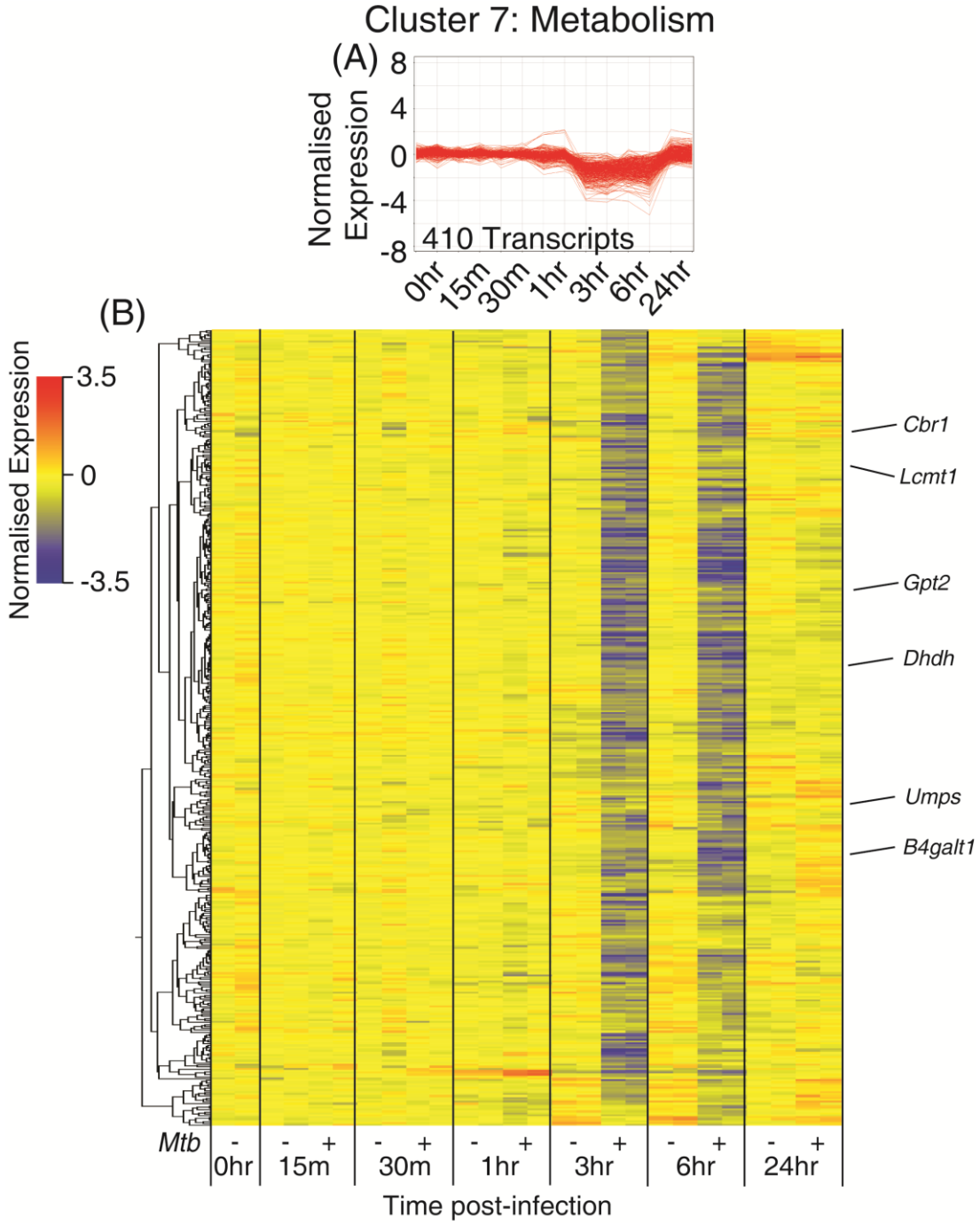

Figure 3.13. Cluster 7 is associated with metabolism. (A) Expression profile of cluster 7. (B) Transcripts in cluster 7 were clustered hierarchically using Pearson centred distance metric with complete linkage.

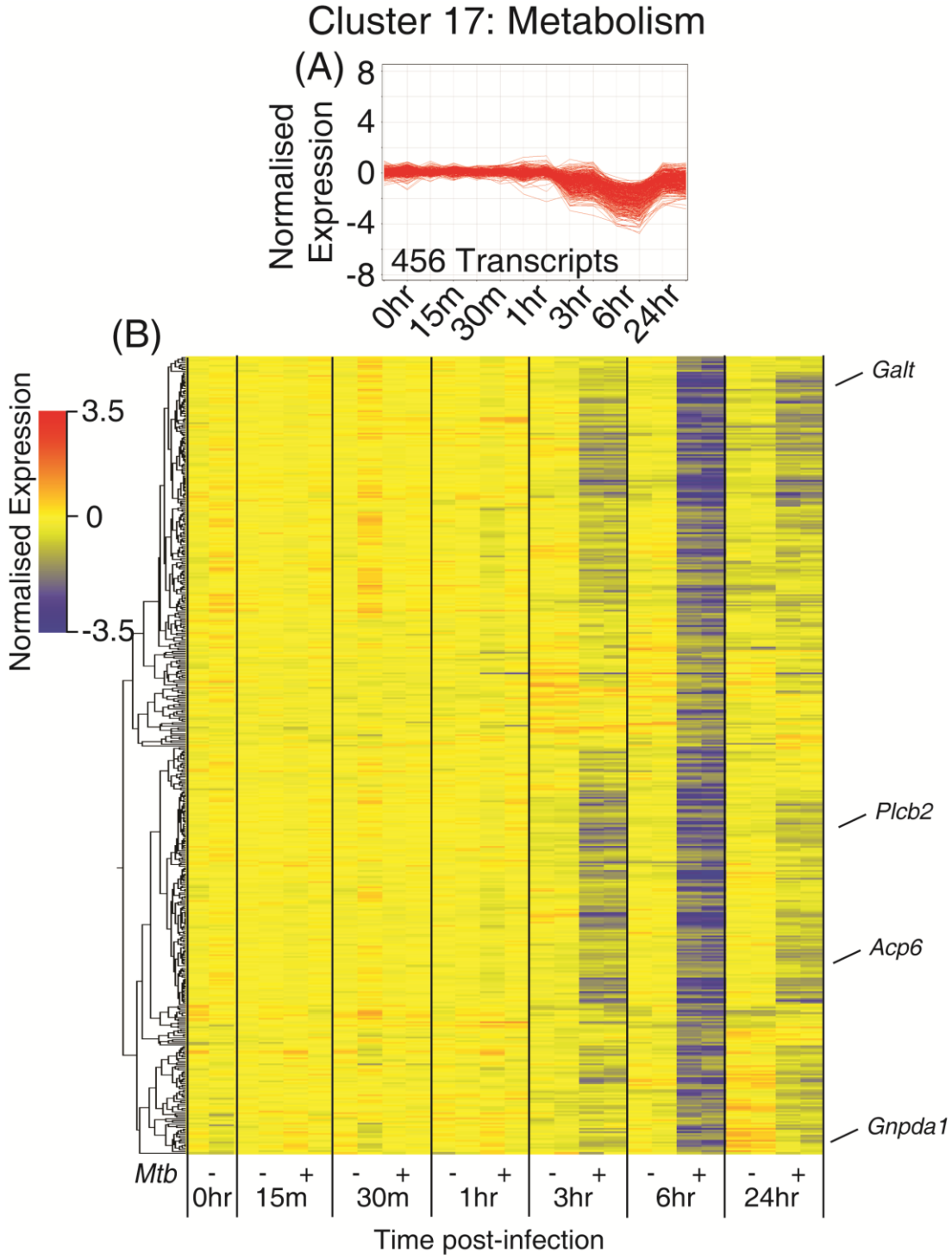

Figure 3.14. Cluster 17 is associated with metabolism. (A) Expression profile of cluster 17. (B) Transcripts in cluster 17 were clustered hierarchically using Pearson centred distance metric with complete linkage.

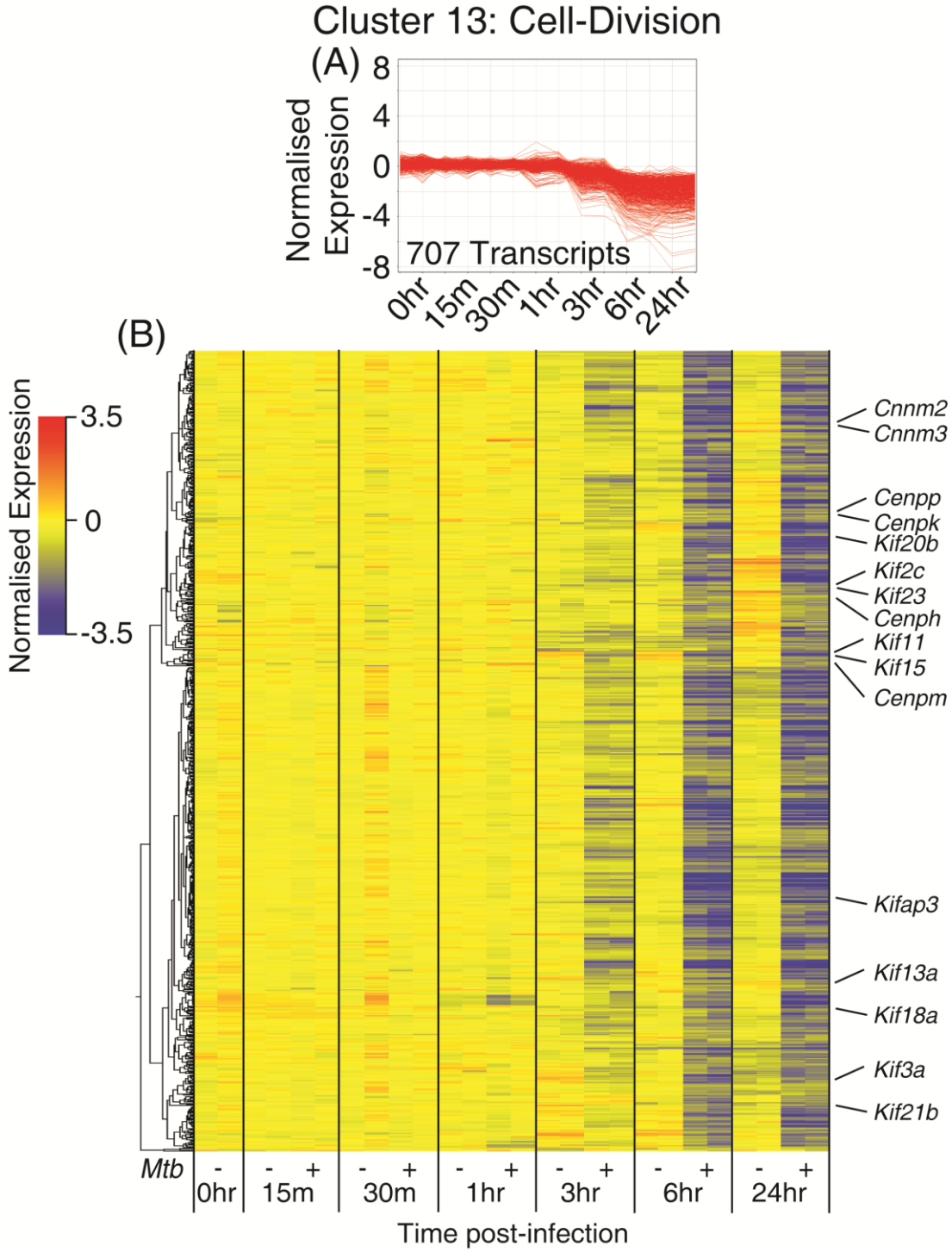

Figure 3.15. Cluster 13 is associated with cell-division. (A) Expression profile of cluster 13. (B) Transcripts in cluster 13 were clustered hierarchically using Pearson centred distance metric with complete linkage.

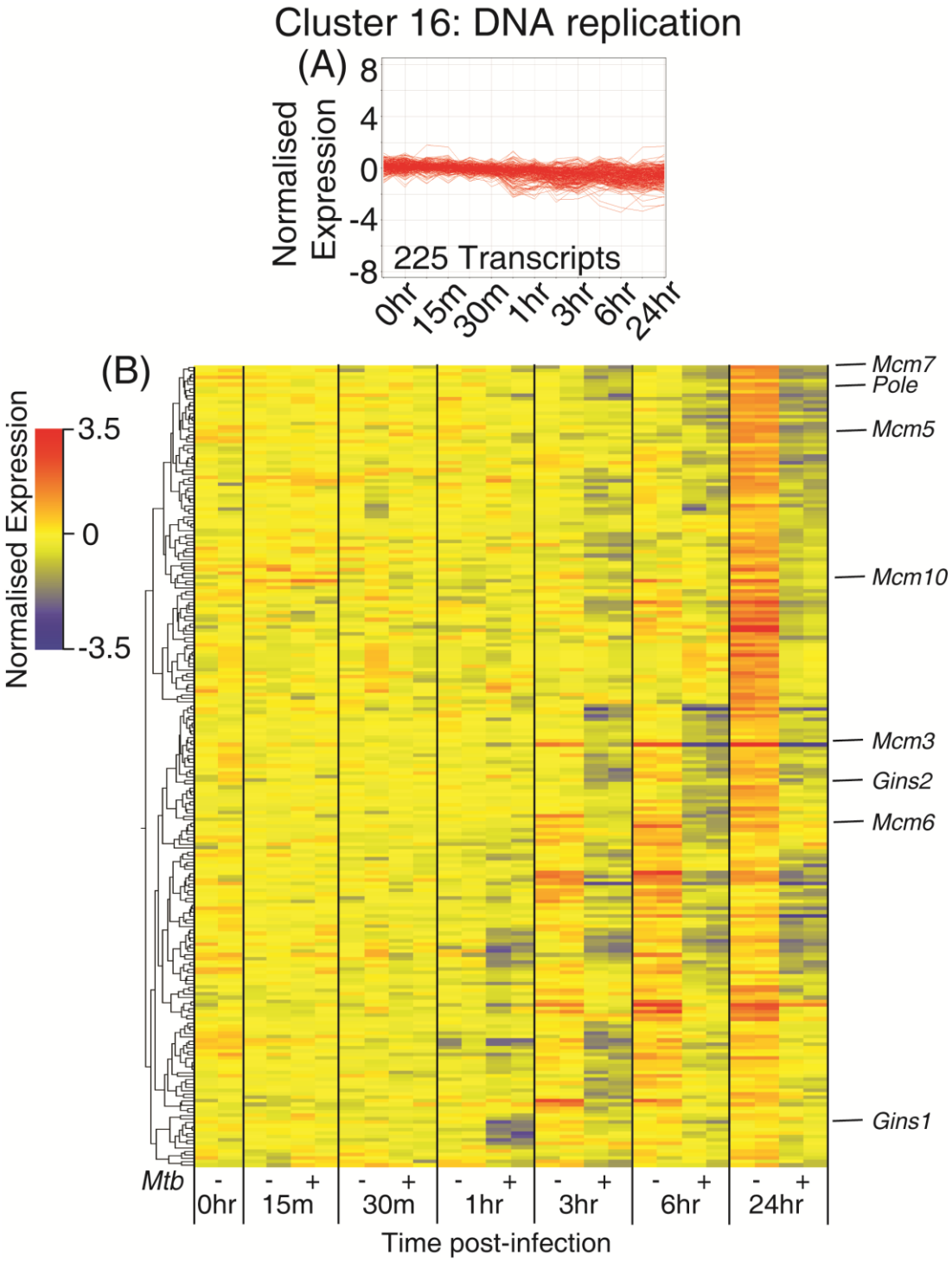

Figure 3.16. Cluster 16 is associated with DNA replication. (A) Expression profile of cluster 16. (B) Transcripts in cluster 16 were clustered hierarchically using Pearson centred distance metric with complete linkage.

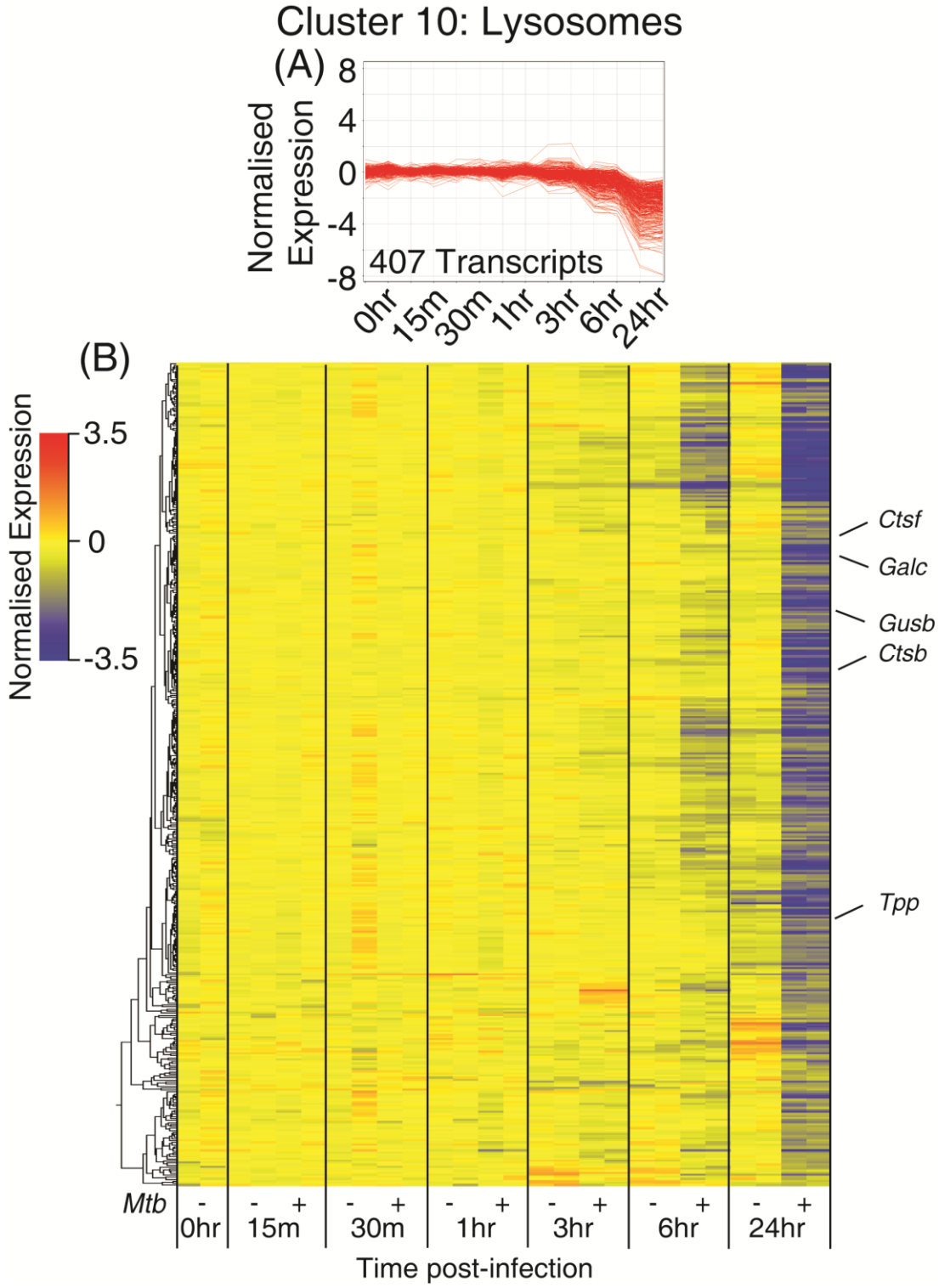

Figure 3.17. Cluster 10 is associated with lysosomes. (A) Expression profile of cluster 10. (B) Transcripts in cluster 10 were clustered hierarchically using Pearson centred distance metric with complete linkage.

3.5. Transcripts present in the 24 *k*-means clusters shown in Figure 3.3

Cluster 0

0610037M15Rik	Eno1	Ly78	Slfn10
1700029F09Rik	Eprs	Ly9	Smox
2010106G01Rik	Faf1	M6pr	Smox
2500002E12Rik	Fam116b	Map3k7	Smox
2810407C02Rik	Fancl	Mlx	Snx2
5730455P16Rik	Flrt2	Mlx	Snx2
AA960436	G3bp2	Mmp9	Sphk2
Abcc5	Gba	Nol3	Stat1
Acp2	Gde1	Nr1h3	Stat6
Acp2	Gipc1	Nr1h3	Symbol
Actb	Glrx	Ociad1	Taf12
Acvrl1	Gm885	P4ha1	Tapbpl
Adam8	Gnat3	Papd4	Tarbp2
Adam8	Gpr35	Papd4	Tbc1d9
Ahcyl1	Gsdmdc1	Parp3	Tcirg1
Ahsa1	Gsta3	Pdcd6ip	Tcirg1
Akt3	Gstm2	Pilra	Tor1a
Alg12	H2-D4	Pilra	Tor1aip1
Asph	H2-K1	Pilrb1	Tpx2
Asph	H2-Q6	Pira4	Trdmt1
Atf2	H2-Q7	Pkm2	Trim21
Atl3	H2-Q8	Pkm2	Tspan3
B230311C12Rik	H2-Q8	Pla2g7	Tspo
B230312A22Rik	H2-T10	Plod3	Tspo
Baz2b	Hcls1	Pmm2	Tyk2
BC002236	Hmgn3	Por	Ube2l6
BC025458	Hmgn3	Pou3f1	Vrk2
Bst1	Hsp105	Psmd2	Wars
C1galt1	Hsp90ab1	Rbm10	Zdhhc24
Calr	Hspa9	Rims3	Zfp313
Cd72	Idua	Rin2	Zubr1
Cdc27	Kif3b	Rnf114	
Cep170	Lgals9	Rnf135	
Cnp	Lin54	Rtn3	
Copg	LOC100044756	Sco1	
Cul2	LOC100045963	Sept8	
Cyfip1	LOC100046056	Sgk3	
D330045A20Rik	LOC209372	Sla	
D430044G20Rik	LOC224487	Slc10a7	
Dedd	LOC229810	Slc11a1	
Dnajc2	LOC230765	Slc11a1	
Dock4	LOC238943	Slc11a1	
Dync1i2	LOC241293	Slc13a3	
EG433633	LOC547150	Slc13a3	
EG630499	LOC624198	Slc28a2	
EG667977	LOC668631	Slc7a7	
Elac2	LOC674135	Slfn10	

Cluster 1

1110004P21Rik	D8Ertd82e	Rasgef1a
1300014I06Rik	D930042A21Rik	Rbm12
1700029G01Rik	Ddx28	Rhof
2600011E07Rik	Elovl6	Rps6ka5
4831426I19Rik	Fam123a	Selp
4831426I19Rik	Fancc	Sh3gl1
4930477M19	Fancc	Shb
4933428G20Rik	Foxp1	Sipa1l2
5033413D16Rik	Gpr132	Slc35e4
5730494J16Rik	Hectd2	Socs2
5830411I20	Hs3st3b1	Specc1
5930403N24Rik	Irgq	Swap70
6430527G18Rik	Itpkc	Swap70
9130604K18Rik	Klf5	Tal1
Adrbk2	Lfng	Tgfbr1
Adss	Llgl2	Tiam1
Agfg2	Lmna	Tjp2
AI115600	Lmna	Tjp2
Ankrd28	LOC233400	Tle3
Ankrd28	LOC380617	Tnfrsf12a
Asxl1	LOC380992	Tnfsf14
Atp13a3	LOC383067	Tnfsf14
B130015M16Rik	LOC384646	Tnfsf4
B3gnt7	Lpl	Ttl
B430005K18Rik	Map2k3	Unc119b
Bahd1	Mapkapk3	Wdhd1
BC022687	Mixl1	Whsc1
BC026996	Mixl1	Zbtb32
Bcl2l11	Mmp10	
Bcl2l11	Mpp5	
Bcl2l11	Mpp7	
Bcor	Odc1	
Bcor	Pdgfa	
Bzw2	Pdgfb	
Capn2	Pdgfb	
Capn2	Phldb1	
Capn2	Plat	
Catnb	Plec1	
Cd70	Plekhf1	
Cd80	Plekhg2	
Cdc14a	Plekhg2	
Cdca4	Pmp22	
Cdca4	Prickle3	
Cdk5r1	Pscd3	
Cdk6	Ptbp1	
D130063H01Rik	Rab15	
D230048P18Rik	Rab3il1	

Cluster 2

1110018G07Rik	BC013712	Gpr141	LOC240921
1600014C10Rik	BC023892	Gpsm2	LOC380616
1600014C10Rik	BC023892	Gstt1	LOC380706
1700034P14Rik	BC050811	Gtpbp2	LOC383125
1700041G16Rik	BC094916	Gvin1	LOC435565
1810035L17Rik	Bcl9	Gys3	LOC547380
2210009G21Rik	Bxdc5	H2-M3	LOC623121
2310010B21Rik	C130020C07Rik	H2-Q5	LOC623121
2310033F14Rik	C130026I21Rik	H2-Q5	LOC625360
2410025L10Rik	C130026I21Rik	H2-T10	LOC667370
2810425O13Rik	Cab39l	H2-T10	LOC667776
2810439F02Rik	Casp8	H2-T23	LOC673556
4921517J08Rik	Casp8	H2-T24	Mad
4930555L11Rik	Ctsc	Hdlbp	Mid1
4930599N23Rik	Ctsc	Homer1	Mlkl
4932438A13Rik	D11Lgp2e	Hook2	Mlkl
4932441K18Rik	D14Ertd668e	Hrh2	Morc3
4933407C03Rik	D14Ertd668e	Ifi44	Mov10
4933412E12Rik	D17Wsu92e	Ifit2	Ms4a6c
5031414D18Rik	Dbnl	Ifit2	Ms4a6d
5033414K04Rik	Dck	Ifit3	Ms4a6d
5133401N09Rik	Ddx58	Ifit3	Mx1
5830484A20Rik	Ddx60	Igsf9	Naip2
6330578E17Rik	Dennd1a	Il18	Oas1b
6430402L23Rik	Dhx58	Il18	Oas1b
6430573D20Rik	Dhx58	Irf2	Oas1g
A330042I21Rik	Dpysl2	Irf7	Oas1g
A530023O14Rik	Dusp28	Irf9	Oas1g
A530032D15Rik	E230011J22Rik	Isg20	Oas2
A530083B17Rik	EG240327	Keap1	Oas3
Adap2	EG240327	Keap1	Oasl1
Adar	EG432555	Keap1	Oasl1
Adar	EG665378	Keap1	Oasl1
Aftph	Eng	Lgals8	Oasl2
AI451617	Enpp4	Lgals8	Oasl2
Aldh1b1	Epsti1	Lgals9	OTTMUSG00000016644
Ankle2	Epsti1	Lgals9	P2ry13
Aoah	Fam162a	LOC100039742	Pgm2
Apba3	Fbxw17	LOC100040462	Pgm2
Arid4a	Fcgr1	LOC100043347	Phc2
Asb13	Fez2	LOC100044190	Phf11
Ascc3	Frmd4a	LOC100044190	Phf11
Ass1	G3bp2	LOC100046469	Plekha4
Atp7a	Gbp10	LOC100046469	Plod3
AU022870	Gm885	LOC100046469	Pml
Azi2	Golga3	LOC100047052	Pml
B430306N03Rik	Golga3	LOC234360	Pml

Cluster 2 Continued	
Prpf4	Trafd1
Psmb8	Trafd1
Psmb8	Trafd1
Psmb9	Trim34
Pttg1	Trim34
Pvrl2	Tyki
Pvrl2	Ube1l
Raf1	Usp18
Rbl1	Vps11
Rbm43	Vps37b
Rilpl1	Vti1a
Rnf213	Whsc1l1
Rnf214	Whsc1l1
Rnf34	Zbp1
Rps4y2	Zbp1
Samhd1	Zcchc2
Samhd1	Zcchc6
Scarf1	Zfp821
Sf1	Zfp821
Sfxn2	Znfx1
Sgcb	
Slc25a12	
Slfn1	
Slfn9	
Smc5l1	
Smc5l1	
Smpdl3b	
Snd1	
Snx2	
Sp100	
Sp100	
Sp140	
Srgap2	
Stard3	
Stard3	
Stat1	
Stat2	
Stx16	
Tagap	
Tap1	
Tbc1d13	
Tdrd7	
Tlr3	
Tmem184b	
Tnfrsf14	
Tnfsf10	
Tpst1	

Cluster 3		
1600021P15Rik	Gm527	Rcsd1
2410081M15Rik	Gmds	Rhbdf1
2610204M08Rik	Gpr137b	Rin3
2700060E02Rik	Gpr176	Rnu65
4632419I22Rik	H2afz	Rplp0
4632427E13Rik	H3f3b	Rrad
5430440L12Rik	Herpud1	Rtn1
5830411K21Rik	Herpud1	Rtn1
6720406L13Rik	Hmgb2	Rtn1
8030481K01Rik	Hopx	Scap
A130062D16Rik	Ifitm1	Slc39a10
A130065C13Rik	Ifitm1	Slc44a1
Actl6a	Ifitm1	Smyd2
Adamtsl4	Igf2bp3	Snora65
Adcy3	LOC242703	Snora70
Apex1	LOC268569	Snx25
Apoe	LOC675899	Stbd1
Atp13a3	Lpl	Tardbp
B3Gat3	Mad2l1	Tmem150
B4galnt1	Mad2l1	Tmem176a
Bex2	Man1a	Tmem176b
Bzw2	Map1lc3a	Tmem55b
C130078A06Rik	Mapre2	Trim3
Ccl6	Mapre2	Trps1
Cd109	Mgea5	Tubb2b
Cd9	Mki67	Ube2n
Cfl1	Mt1	Uck2
Chst7	Ncl	Uck2
Clec4b1	Nde1	Uck2
Crebl2	Ndrg1	Zadh2
D12Ertd647e	Ndrl	
D12Ertd647e	Nelf	
D330028D13Rik	Nelf	
Dgka	Nola3	
Dgka	Olfm1	
Dhcr24	Olfm1	
Dhx9	Olfm1	
Dpep2	Pam	
Dpep2	Pif1	
E130012A19Rik	Pkd2	
E430031D18Rik	Pkmyt1	
Eef1d	Ppic	
Emp2	Ppic	
Fin15	Ptpla	
Fkbp5	Purb	
Galnt10	Pusl1	
Gdpd1	Rbbp4	

Cluster 4

1100001G20Rik	Atp6v0a2	Dnclc1	Hsp90aa1
1110008F13Rik	Azi2	Dock4	Hspa1a
1110008F13Rik	B130017M24Rik	Dst	Hspa1b
1700095J19Rik	B4galt7	Dtnb	Hspd1
1700112C13Rik	Bak1	Dync1h1	*Ifnb1*
1700112E06Rik	BC021438	Dync1i2	Igfbp7
1700123O20Rik	BC067047	Dynll1	Il15ra
1810055G02Rik	Bfar	Ece2	Il2rg
2010209O12Rik	Bfar	Edg5	Il7
2210009G21Rik	Bxdc1	EG432448	Inhba
2210019E14Rik	C1galt1c1	EG433182	Irak3
2400010D15Rik	C3	EG622339	Irf5
2810405F04Rik	C330023M02Rik	EG630499	Itgal
2810439F02Rik	Casp1	Egln3	Kif1b
3110050N22Rik	Cbwd1	ENSMUSG00000053338	Klc1
4930518I15Rik	Ccdc59	Erap1	Kpna6
4930556B16Rik	Ccng1	Esd	Lamp2
4933430I17Rik	Cd180	Esd	Lamp2
5730596K20Rik	Cd200	Esd	Larp5
9130005N14Rik	Cd200	Esrra	Layn
9430043O10Rik	Cd200	Evl	Ldha
9630025H16Rik	Cenpj	F630107D10Rik	Lemd2
9930022F21Rik	Cept1	Fam164a	Lnpep
A630001G21Rik	Cfb	Fancl	LOC100043796
AA589481	Chordc1	Fpr1	LOC100044215
Aars	Clip2	Gbp2	LOC100044948
Abcf2	Cndp2	Gca	LOC100044968
Abhd2	Cnih4	Gclm	LOC100045967
Abhd2	Cox17	Glipr2	LOC100046039
Abhd2	Cox18	Glrx1	LOC100046163
Abr	Cox18	Gmppb	LOC100046163
Acot7	Creld2	Gng12	LOC100046469
Acot7	Creld2	Gpr31c	LOC100048554
Acp2	Crem	Gsdmdc1	LOC193533
Adar	Cst7	Gsdmdc1	LOC194744
Afp	Ctsz	Gtf2a1	LOC219049
Akt3	Ctsz	Gtf2f1	LOC223653
Aldoa	Cugbp1	H2-K1	LOC224163
Ankrd37	Cycs	H2-M2	LOC234882
Armc7	Cycs	Hat1	LOC245892
Arv1	Cycs	Hax1	LOC381947
Arv1	D0H8S2298E	Hdc	LOC384343
Ascc2	D230004N01Rik	Hk3	LOC385905
Asns	D4Ertd22e	Hmox1	LOC386067
Asns	D630022O22Rik	Hn1	LOC634015
Asns	Dnajb11	Hp	LOC671434
Atl3	Dncic2	Hsf2	LOC677317

Cluster 4 Continued

Lrp11	Plekhf2	Psme4	Sra1
Magohb	Polb	Ptges	Srpr
Marco	Por	Ptk2b	St3gal5
Mdh2	Por	Ptpn6	Stau2
Med7	Ppa1	Ptrh2	Stx3
Mif	Ppap2a	Rab21	Tapbpl
Mif	Ppap2a	Rassf1	Tarbp2
Mir16	Ppap2a	Rassf1	Tfg
Mitd1	Ppm1h	Rchy1	Tmbim4
Mkks	Ppp1r11	Rilpl1	Tmem120a
Mmp25	Ppp1r11	Rin2	Tmem171
Mod1	Prdx1	Rnf135	Tmem49
Mrpl39	Prdx1	Rnf135	Tmem68
Mrps10	Prdx5	Rnf31	Tmem77
Mrs2	Prdx5	Rrbp1	Tpi1
Mthfd2	Prkrip1	Rrbp1	Tpi1
Nckap1	Prkrip1	Rtn2	Trem3
Nln	Procr	Rtn3	Treml4
Nlrc4	Prokr1	Saa1	Treml4
Nmi	Prpf38a	Sco1	Tsg101
Nmt1	Psma4	Scoc	Tspan3
Noc4l	Psma7	Sdc1	Tspo
Nos2	Psmb2	Sepw1	Tssc1
Npc1	Psmb2	Serpinb2	Ttc1
Npc2	Psmb3	Sh3bp2	Ttc9c
Npy	Psmb5	Siat9	Ttc9c
Nqo1	Psmb7	Sipa1l1	Txn1
Nus1	Psmb7	Skap2	Ubap1
Osbpl3	Psmb7	Slc11a1	Ube2k
Osbpl3	Psmc1	Slc12a3	Ube2m
Otud3	Psmc2	Slc16a3	Uchl1
P4ha1	Psmc2	Slc22a4	Upk3b
P4ha2	Psmc4	Slc25a10	Upp1
Pcsk7	Psmc4	Slc35b1	Upp1
Pdia6	Psmc5	Slc39a7	Upp1
Pdzd11	Psmd1	Slc3a2	Usp25
Pdzd11	Psmd1	Slc7a2	Wdr68
Pdzd11	Psmd1	Slc7a8	Xkr8
Pex11a	Psmd11	Slc7a8	Zfp710
Pfkl	Psmd11	Slco3a1	Zkscan6
Pgam1	Psmd12	Smg7	Zmynd15
Pgk1	Psmd12	Smurf1	
Phf20l1	Psmd14	Snx11	
Pik3cb	Psmd14	Snx16	
Pkp4	Psmd7	Snx20	
Pkp4	Psme1	Soat2	
Plekha2	Psme1	Sra1	

Cluster 5

1500010G04Rik	Capzb	F2r	LOC100040592
2300006M17Rik	Car2	F730011O15Rik	LOC100044177
2310007G05Rik	Ccdc93	Fam13b	LOC100045280
2410015C20Rik	Ccdc94	Far1	LOC100045280
2610027C15Rik	Ccl24	Fcgr2b	LOC100045343
2610103J23Rik	Ccl9	Fchsd2	LOC100045439
2610304I02Rik	Ccnyl1	Fgr	LOC100046025
2900057D21Rik	Ccrn4l	Foxp4	LOC100047856
2900073G15Rik	Ccrn4l	Furin	LOC100048221
3110003A17Rik	Cd244	Furin	LOC100048706
3202002H23Rik	Cd44	Fzd1	LOC100048721
4832412D13Rik	Cd44	Fzd5	LOC381066
4932442L11Rik	Cd44	G3bp1	LOC385032
4933424B01Rik	Cd74	Gda	LOC677008
4933432P15Rik	Cd74	Gfi1	Map3k6
8030402P03Rik	Cd82	Gng4	Mapkapk2
9130223C08Rik	Cdc37	Gnl3	Mapkbp1
9430008C03Rik	Cdc42ep4	Grwd1	Mark2
9430080K19Rik	Cdkn2a	Gtpbp4	Mat2a
A430093F15Rik	Cdv3	H2-Aa	Mat2a
A530026H04Rik	Cdv3	H2-Eb1	Mcf2l
Acat2	Chst11	Hivep3	Mesdc1
Acot10	Ciita	Hivep3	Mfsd7c
Actn1	Cobll1	Hnrnph1	Mkl2
Adam17	Cp	Hnrpab	Mkl2
Adam17	Cstf2	Hnrpll	Mobkl2c
Adamts4	Cx3cl1	Hrb	Mon1b
Aebp2	Cyp51	Htra4	Mrgpra2
Aebp2	D11Moh35	Icosl	Mtmr14
Alcam	D130004H04Rik	Idi1	Mtmr14
Alkbh	D230007L07Rik	Igf2r	Mtmr14
Anxa1	D430020J02Rik	Igsf6	Myadm
Anxa3	Dcbld2	Il13ra1	Mycl1
Anxa3	Dcun1d5	Il17rd	Myo10
Anxa5	Ddx21	Il4i1	Myo10
Appl1	Dlg3	Ing5	Myo10
Aqp9	Dmtf1	Insig1	Myo1g
Arnt	E130014J05Rik	Ipo5	Narg1
B230334I05Rik	E430028B21Rik	Itgb1	Ncoa5
B630005N14Rik	Ednrb	Jdp2	Net1
BC046404	Ednrb	Jmjd2a	Nfe2l2
Brp17	Ednrb	Jmjd2a	Nfkb1
C230043G09Rik	Eid3	Kpna3	Nfkb1
C330006P03Rik	Eif1a	Kremen	Nola2
Cacnb3	Eif2c3	Lad1	Notch1
Calcrl	Emp1	Lck	Nupl1
Camkk2	Erdr1	Ldlr	Oplah

Cluster 5 Continued

Optn	Six1	Tsc22d1
Optn	Slc11a2	Tshz1
Paf1	Slc11a2	Ubtd2
Pcdh7	Slc12a4	Ubtd2
Pcdh7	Slc2a6	Ubtd2
Phldb1	Slc30a4	Vasn
Plec1	Slc39a14	Wdr92
Plekhg2	Slc39a14	Wsb2
Plekho2	Slc41a2	Zbtb33
Pogk	Smcr8	Zdhhc21
Ppp1r10	Snn	Zfp263
Ppp2cb	Snn	Zhx2
Ppp4r1	Spag9	Zic4
Prdm1	Spag9	Znrf1
Prkcd	Spata13	Zwint
Ptbp1	Sphk1	
Ptbp1	Sqle	
Ptpn1	Sqle	
Ptrf	Srebf2	
Rab6	Ssr3	
Rac3	St7	
Rai12	Stard7	
Rai12	Stard7	
Rai12	Stat5a	
Rassf4	Stat5a	
Rassf4	Stx6	
Rassf4	Syk	
Rbm12	Syncrip	
Rcl1	Tcfec	
Rela	Tha1	
Reps1	Tirap	
Rffl	Tmem120b	
Rffl	Tmem176a	
Rnf145	Tmtc2	
Rnpc3	Tnfaip2	
Rras2	Tnfaip2	
Rrs1	Tnfrsf5	
Rufy3	Tnfrsf6	
Samsn1	Tnfrsf9	
Sbno2	Tnfrsf9	
Scamp1	Tnip1	
Schip1	Tpbg	
scl0001233.1_26	Traf3	
scl0002617.1_582	Trim36	
Sdad1	Trim36	
Sept11	Trim36	
Shisa3	Trip10	

Cluster 6			
1300018I05Rik	Cd274	Gbp3	LOC100047963
1600029O10Rik	Cd69	Gbp5	LOC100048105
2010106G01Rik	Cep350	Gbp6	LOC100048299
2010106G01Rik	Cep350	Gbp6	LOC100048346
2310008I22Rik	Chac1	Ggnbp2	LOC100048556
2310044G17Rik	Chmp4b	Glipr2	LOC100048556
2410013I23Rik	Clec4a1	Glipr2	LOC100048583
2610208M17Rik	Cp	Gng12	LOC271505
2700055A20Rik	Cp	Gss	LOC277707
2700055A20Rik	Crlf3	Gss	LOC385639
4933409L06Rik	Csf3r	Hdc	LOC638301
9030625A04Rik	Csf3r	Hk1	LOC667034
9130230N09Rik	Csf3r	Hp	LOC668492
A030007L17Rik	Ctsc	Hp	LOC674706
A430084P05Rik	Cxcl10	Ifi204	LOC675594
A430084P05Rik	Cxcl10	Ifi205	LOC676748
A930026L03Rik	Cxcl9	Ifi35	Lox
AA467197	D17Wsu92e	Ifi47	Lsm12
AA960436	D17Wsu92e	Ifih1	Lsm12
Acsl1	D430007J11Rik	Ifitm1	Ly9
Adar	D4Wsu132e	Ifrg15	Lyrm1
AI451557	Daxx	Igtp	Mad
AI607873	Dcp2	Iigp2	March1
Aim1	Ddx24	Il15ra	Mfsd7
Aim2	Ddx24	Il15ra	Mfsd7a
Anxa4	Dlm1-pending	Il15ra	Mizf
Anxa6	Dtx3l	Il20ra	Mnda
Arid5b	E330016A19Rik	Iqsec2	Mthfd2
Arl4a	Eif2ak2	Irgb10	Mvp
Ascc3	Eif5	Irgm1	Mx2
Atl3	Etnk1	Irgm1	Nab1
Atp10a	Etnk1	Irgm1	Nampt
B230339H12Rik	F7	Jak3	Nod1
Bambi-ps1	F730045P10Rik	Klhl5	Nr3c1
Batf2	Fam20b	Lamp2	Nrap
C230055K21Rik	Fcgr2b	Lass6	Nt5c3
C330023M02Rik	Fcgr2b	Lcn2	Oas1a
C330023M02Rik	Fcgr2b	LOC100038830	Oas1g
C330023M02Rik	Fgl2	LOC100038882	Ogfr
Caprin1	Fmo3	LOC100041137	Otud5
Capza2	Fpr2	LOC100044430	Oxsr1
Cbl	Frag1	LOC100045567	Parp12
Ccdc25	Frag1	LOC100045644	Parp14
Ccdc50	Fyb	LOC100046393	Parp14
Ccdc59	Gbp2	LOC100047619	Parp8
Ccdc86	Gbp2	LOC100047707	Pcgf5
Ccl5	Gbp3	LOC100047963	Peli1

Cluster 6 Continued	
Peli1	Sp100
Pex11a	Stx4a
Pik3ap1	Taar3
Pilra	Tap2
Pira1	Tapbp
Pira11	Tapbpl
Plekhf2	Tcirg1
Plekhm3	Tcof1
Plekhm3	Tgfbi
Pmm2	Tgfbi
Pols	Tgs1
Ppp3cc	Tgtp
Prpf38a	Tm9sf4
Prpf38a	Tm9sf4
Prpf4	Tor1aip1
Ptprc	Tor1aip2
Rab3d	Tor3a
Rab8a	Trex1
Rac2	Trex1
Ralb	Trim21
Rap2c	Trim26
Rap2c	Trim30
Rapgef2	Ube2l6
Rasa4	Ubg
Rasa4	Usp18
Rhbdd2	Wars
Rin2	Wars
Rp23-357i14.1	Wdfy1
Rsad2	Zfp295
Rtp4	Znfx1
Samd8	Zubr1
Samd9l	Zwint
Sav1	
Scl30a1	
Sclo30a1	
Sema4a	
Serpina3f	
Serpina3g	
Setdb2	
Setdb2	
Sh3bp5	
Slamf8	
Slc30a1	
Slco3a1	
Slco3a1	
Slfn5	
Smg7	

Cluster 7

0610009O20Rik	5530400K22Rik	BC088983	Cyb561d1
0610042C05Rik	5730409G15Rik	Bcdin3d	D15Wsu169e
1110034A24Rik	5730478M09Rik	Bola1	D430028G21Rik
1110034A24Rik	5830482F20Rik	C030002B11Rik	Dgcr6
1190007I07Rik	5930434B04Rik	C030026E19Rik	Dgkz
1200013B08Rik	5930434B04Rik	C130050O18Rik	Dguok
1300018I17Rik	6330503K22Rik	C130065N10Rik	Dhdh
1500031H01Rik	8430432M10Rik	C2cd2l	Dhdh
1500031L02Rik	9330129D05Rik	C2cd3	Dhx34
1500041N16Rik	9330134C04Rik	C530028I08Rik	Dhx57
1700022C21Rik	9330175B01Rik	C820007E08Rik	Disp1
1700023M03Rik	9330177P20Rik	C87436	Dmwd
1700030J22Rik	9630031D17Rik	Casp9	Dmwd
1700030K09Rik	9630058J23Rik	Cbr1	Dnmbp
1700065O13Rik	A130010J15Rik	Ccdc111	Dnmt1
1810009O10Rik	A330057G13Rik	Ccdc111	Dok2
1810015C11Rik	A830007P12Rik	Ccdc123	Dscr3
1810043G02Rik	AB112350	Ccdc123	Dscr3
1810043G02Rik	Abcd1	Ccdc28b	Dusp19
1810048J11Rik	Abcd4	Ccdc28b	Dusp6
2010003O18Rik	Acad8	Ccdc45	Dusp6
2210011G09Rik	Acad8	Ccdc77	E2f1
2210012G02Rik	Adck4	Ccdc97	EG240038
2210018M11Rik	AI256775	Ccnd1	EG433224
2210021J22Rik	AI467606	Ccnd1	Endogl1
2210410D02Rik	Alg2	Ccnd1	ENSMUSG00000054212
2310001H12Rik	Angptl6	Ccnd3	Epb4.1
2310007F21Rik	Apobec1	Ccrk	Epb4.1l1
2310007F21Rik	Arhgap25	Ccrk	Epb4.1l1
2310007F21Rik	Arhgap27	Cdc2l6	Etv1
2310047B19Rik	Arhgef18	Cecr5	Fam120b
2310079N02Rik	Arl16	Chchd5	Fblim1
2400009B08Rik	Arl6	Chchd8	Fbxl4
2410004L22Rik	Arrdc3	Chek2	Fbxo31
2410017P07Rik	Atf7ip	Chek2	Fbxo9
2410018C17Rik	Atmin	Chst12	Fgd4
2410019G02Rik	Atp5s	Cisd1	Fgd4
2410075B13Rik	B230333C21Rik	Cmas	Fli1
2610010A15Rik	B3galnt1	Cml1	Gab3
2610019A05Rik	B4galt1	Coasy	Gab3
4732460K03Rik	Bbs5	Cog7	Gapt
4930430F08Rik	BC016495	Coq10a	Gatc
4930455F23Rik	BC021790	Coq4	Gdf9
4930455F23Rik	BC032203	Cpeb3	Gemin6
4930455G09Rik	BC044804	Creb3	Gm169
4932415G12Rik	BC049806	Creb3l3	Gorasp1
4932415G12Rik	BC066140	Csk	Gpt2

Cluster 7 Continued

Gsg2	Mettl8	Pdlim2	Setdb1
Gtf3c1	Mfsd9	Per1	Sfi1
Gtrgeo22	Mlh3	Per1	Sfrs17b
H6pd	Mmab	Phf17	Sfxn4
Hdac5	Mocos	Phf20	Sip1
Helb	Mon1a	Pik3r2	Sipa1
Helb	Mppe1	Pms2	Sirt4
Hexdc	Mpv17l	Pnpla6	Skp2
Hhex	Mrpl2	Ppcs	Slc12a9
Hip1	Mrps35	Ppm2c	Slc25a38
Hmg20a	Mtrf1	Ppp1r3b	Slc25a45
Htr2b	Myo7a	Prim2	Slc29a3
Ift88	Narf	Psg23	Slc35c2
Inpp5d	Nbr1	Ptger2	Slc38a9
Irf2bp2	Ncrna00117	Ptpn7	Slc45a4
Katnb1	Nek1	Pwwp2b	Slc8a1
Kbtbd7	Nfatc1	Rab11fip3	Smad3
Kbtbd7	Nfatc1	Rab19	Smad3
Kif13b	Nfic	Rab19	Smarcal1
Kif5c	Nfic	Rab27a	Smyd4
Klhdc1	Nlrx1	Rab39	Snx30
Klhl22	Nlrx1	Rab3a	Spg20
Lancl1	Nme7	Ralgps1	Spsb2
Lbh	Nme7	Rasl2-9	Stambpl1
Lbh	Nme7	Rassf2	Synj2
Lcmt1	Nte	Rbbp9	Taf5
Lcmt1	Nudt1	Rbbp9	Taf6
Limk2	Nudt22	Rfc1	Tcte3
LOC100043555	Obfc2a	Rftn2	Tdrd3
LOC100044103	Ociad2	Rftn2	Tex2
LOC100044298	Ociad2	Rhobtb2	Tex2
LOC100044468	Orai3	Rmnd1	Tg737Rpw
LOC100044636	Orc5l	Rpap1	Thnsl1
LOC100045981	Orc5l	Rpap2	Tial1
LOC226486	Osgepl1	Rpap3	Tmem129
LOC676420	Oxr1	Rprd1a	Tmem218
Lrrc20	Paox	Rps6ka1	Tmem218
Lrrk1	Paox	Rrm2b	Tmem86a
Lrrk1	Paox	Rwdd2b	Tnfaip8l2
Map3k12	Pard6a	Samd1	Tnfrsf13b
Map3k7ip1	Pard6a	Saps2	Tnrc6c
Map3k7ip1	Pbx2	Sbk	Trim45
Mapk1ip1	Pcgf1	scl0001487.1_50	Tsc22d3
Mcph1	Pddc1	scl0015365.1_6	Tsc22d3
Mcph1	Pdlim2	Sec22c	Tsen2
Mertk	Pdlim2	Senp7	Ttc5
Mertk	Pdlim2	Sesn2	Ttf2

Cluster 7 Continued
Tusc4
Txnip
Tyw1
Uaca
Ubac1
Ubfd1
Uhrf1bp1
Uhrf1bp1
Umps
Unc119
Uvrag
Vars2
Vegfa
Vps26b
Vps33b
Wdr60
Wdr60
Wdr76
Wipf1
Zbtb1
Zbtb24
Zc3h6
Zc4h2
Zfp386
Zfp41
Zfp41
Zfp467
Zfp512
Zfp512
Zfp688
Zfp825
Zfyve26
Zranb3
Zw10

Cluster 8		
1110031I01Rik	Hist2h3c1	Tgif1
2310016C08Rik	Ibrdc3	Tgif1
2310016C08Rik	Id2	Tmem49
4930431B09Rik	Id2	Tmem49
5430427O19Rik	Ifrd1	Tmem49
5830435K17Rik	Il10	Tnf
6030408C04Rik	Il1b	Tnf
9130227N12Rik	Il2rg	Tnfaip3
A530093H06Rik	Ireb2	Tnfsf9
Atf3	Irf1	Traf6
Atl2	Irf1	Zfp36
Atl2	Irf1	Zrsr2
Axud1	Irf1	
B230120H23Rik	Irf1	
BC031781	Jmjd3	
Ccdc21	Junb	
Ccl2	Kctd12	
Ccl3	Klf6	
Ccl4	Klf6	
Ccl4	LOC100046232	
Ccl7	LOC100046406	
Ccl7	Lrp12	
Ccnl1	Maff	
Ccrl2	Map3k8	
Cebpb	Mib1	
Coq10b	Myd116	
Coq10b	Nfkbia	
Cxcl1	Nfkbia	
Cxcl2	Nfkbiz	
Cxcl2	Pde4a	
Cybb	Phlda1	
Dicer1	Pim1	
Dusp2	Plk2	
Dusp2	Plk3	
Dusp4	Pvr	
Dyrk2	Rasgef1b	
EG622976	Rasgef1b	
Ets2	Rgs1	
Gadd45b	Rgs1	
Gadd45b	Rgs1	
Gdf15	Rusc2	
Gm129	Sgk1	
Gm129	Slc25a33	
Gp49a	Socs3	
H3f3b	Socs4	
Hist1h3h	Tbc1d10a	
Hist2h3b	Tgif1	

Cluster 9

0710001C05Rik	Azi2	Hist2h2aa1	Mocs1
1110038F14Rik	B430201A12Rik	Hist2h2aa2	Morf4l2
1110038F14Rik	B430201A12Rik	Hist2h2ac	Mscp
1110059G02Rik	Bat5	Hsd17b7	Mvd
1700013A01Rik	Cacybp	Hspa5	Nampt
1700085B03Rik	Ccdc88b	Hspa8	Ncf4
1810009K13Rik	Cct7	Htr4	Nkiras1
1810035L17Rik	Cd69	Hvcn1	Noc4l
2210407N10Rik	Clcn7	Hvcn1	Nox1
2310061F22Rik	Clec5a	Ifitm3	Nsdhl
2610002M06Rik	Clec5a	Ifna6	Nsdhl
2610103J23Rik	Clecsf5	Il15	Nt5dc3
2810021O14Rik	Copg	Ints12	Nubp1
2810430B18Rik	Csda	Kars	Nubp1
2900045G02Rik	Dapk1	Lass6	OTTMUSG00000000971
3830402I07Rik	Dcbld2	Lass6	Panx1
4631409F12Rik	Dgat2	Lig3	Panx1
4732458O05Rik	Dld	Lims1	Papss2
4930453N24Rik	Dnajb6	LOC100045999	Pcyt2
4930599N23Rik	Dusp11	LOC100047184	Pdia5
5330426P16Rik	E330016A19Rik	LOC100048105	Pdss1
5730508B09Rik	Eapp	LOC100048105	Pgam1
5730589K01Rik	Edf1	LOC100048803	Pgbpll
6330416G13Rik	Efhd2	LOC235997	Pgd
9530018I07Rik	EG632248	LOC236223	Phtf2
A030007L17Rik	Eif4g2	LOC380756	Pik3r6
A530088I07Rik	Ep400	LOC383293	Pik3r6
AA691260	Esrra	LOC384104	Plekhf2
Aacs	Exosc10	LOC385662	Pmvk
Abca7	Expi	LOC621823	Pno1
Acot9	Fabp5	LOC624784	Pnp1
Adamtsl5	Fbxo18	LOC637082	Pnpt1
Adar	Fcgr2b	LOC638034	Pofut1
Adhfe1	Fdps	LOC665181	Ppm1k
Adrbk1	Fmnl2	LOC666559	Ppm1k
Agrn	Gm941	LOC666652	Ppm1k
Agtrap	Gm960	Lsm12	Prpf38a
Aig1	Gna15	Lss	Psma5
Aim2	Gphn	Mafk	Psmb10
Alkbh5	Gphn	Map2k1	Psmb10
Amdhd1	Gphn	March1	Psmb6
Ankrd17	Gpr126	March10	Psmd6
Anp32a	Gss	Mdfic	Psmd8
Anxa4	Gtpbp6	Mdk	Psme2
Atp6v1d	Herc1	Mina	Ptprc
AW146242	Hisppd1	Minpp1	Rab1
Axl	Hist2h2aa1	Mmp2	Rab9

Cluster 9 Continued	
Ranbp2	Vps26
Rbm19	Vps29
Rfwd2	Vps54
Rtn4r	Wdr43
Rwdd1	Whdc1
Sap30	Wtap
Sc4mol	Xrn2
Scfd1	Ythdf1
Scrn1	Ywhaz
Seh1l	Zbtb5
Sh3kbp1	Zdhhc5
Sh3kbp1	Zfp213
Siat7b	Zfp319
Slc23a2	Zfp622
Slc25a37	Zfp710
Slc25a44	Zyx
Slc31a1	
Slc35f5	
Sltm	
St13	
St18	
St3gal3	
Stard5	
Stard5	
Stx4a	
Surf4	
Taf15	
Tbrg4	
Tcirg1	
Tcof1	
Timm17a	
Timm8a1	
Tmem128	
Tmem77	
Tmsb10	
Tnfsf15	
Tomm70a	
Tor1aip2	
Tpst2	
Traf5	
Txndc4	
Ube2e2	
Ubl4	
Ush2a	
Usp25	
Vcan	
Vcpip1	

Cluster 10			
1110003F05Rik	Ak3	Cdkl2	EG629383
1190002F15Rik	Aldh16a1	Cdkl2	EG633570
1700052O22Rik	Aldh2	Cdkn3	Eif4b
1700054E11Rik	Aldh5a1	Cenpa	Emb
2210008N01Rik	Ang	Centd3	Emr4
2210013O21Rik	Angptl2	Chi3l3	ENSMUSG00000043795
2210039O17Rik	Anln	Cib2	Ephx1
2210411K11Rik	Anp32e	Cib2	Epm2aip1
2300002D11Rik	Aof1	Ckap2l	Epm2aip1
2310003H01Rik	Aph1b	Clec4b1	F13a1
2310004I03Rik	Aplp2	Clec7a	F630001K14Rik
2310047M10Rik	Apoa2	Clecsf12	Fah
2310047M10Rik	Apoc1	Copz2	Fam129a
2610019E17Rik	Apoc1	Copz2	Fam131a
2810025M15Rik	Arhgap6	Copz2	Fam132a
2810417H13Rik	Arhgap6	Copz2	Fam168b
2900093K20Rik	Arpp19	Cox7a2l	Fbxw8
3000003G13Rik	Arsb	Csad	Fcgrt
3110007F17Rik	Atg16l2	Csf1r	Fcgrt
3110013H01Rik	Atg9b	Csf1r	Fcna
3300005D01Rik	Atp2a3	Ctns	Fcrls
4933421H10Rik	Atp2a3	Ctsb	Fgf13
5133400G04Rik	Atp6v0a1	Ctsf	Fh1
5133400G04Rik	Atpif1	Cyp27a1	Fhit
5830434P21Rik	Atxn3	Cyp27a1	Fin14
5830454D03Rik	Aurka	Cyp27a1	Fkbp7
6330509M05Rik	Avpi1	Cyp4f18	Fkbp9
6530406A20Rik	Aytl2	Cyp4f18	Frmd6
6720467C03Rik	Bahcc1	D16Bwg1494e	Fxyd2
6720467C03Rik	BC004728	D6Ertd365e	Gadd45a
7330410H16Rik	BC004728	D830044I16Rik	Gadd45a
8430410A17Rik	Bcas3	D9Ertd392e	Galc
9030418K01Rik	Birc5	Dapk1	Galnt10
9630007E23Rik	Birc5	Dapk1	Gas6
9830001H06Rik	Birc5	Dcun1d4	Gcap27
A130070G01Rik	Blm	Ddb2	Gcat
A930005G04Rik	C230096C10Rik	Diap3	Glb1l
A930024F17Rik	C430049K18Rik	Dicer1	Glg1
Abcb1b	C920004C08Rik	Dnmt3b	Gm1673
Abcb4	Ccnb1	Dpep2	Gm1673
Abi2	Ccnb1	Dtd1	Gng10
Abi2	Cd300a	Eef1b2	Gng10
Aco1	Cdan1	Eef1b2	Gpx3
Adck5	Cdc20	Efemp2	Gstk1
Aff4	Cdc20	EG245190	Gstm5
AI481316	Cdc20	EG245297	Gusb
AI850995	Cdca3	EG382843	H2afx

Cluster 10 Continued

H2-DMa	LOC667005	Nup210	Rpl31
H2-DMa	Lsm6	Nusap1	Rpl4
H2-Ke6	Lsm6	Oip5	Rpo2tc1
Hadhb	Ltbp3	Pabpc4	Rps15
Hexa	Ltc4s	Pabpc4	S100a1
Hist1h2ag	Ltf	Pccb	S100a4
Hist1h4k	Lyzl4	Pccb	S100a9
Hist1h4m	Lztr1	Pcp4l1	Sag
Hsd17b4	Macrod1	Pcp4l1	Satb1
Iap	Maged1	Pdzk1ip1	Scrib
Igf1r	Maml2	Phpt1	Sdf4
Igfbp4	Man2b1	Plk1	Sema6b
Igfbp4	Man2b1	Pon2	Sepp1
Igh-6	Matk	Ppa2	Sepp1
Igh-6	Matk	Prc1	Serinc3
Igsf4a	Mboat1	Prkd3	Serpinb6a
Il6st	Mbtd1	Prkd3	Serpinf1
Immp2l	Mgl1	Prkg1	Sfrs5
Khk	Mgl2	Prodh	Sft2d2
Khk	Mgl2	Pros1	Sidt2
Kif22	Mgst3	Prps1	Sidt2
Kif4	Mgst3	Ptpla	Slc12a2
Klk1b1	Mmd	Ptprs	Slc12a2
Klk1b11	Mprip	Ptprs	Slc22a18
Krr1	Mrc1	Rab34	Slc26a6
Lass5	Msr2	Rab34	Slc29a1
Lbr	Msr2	Rab34	Slc9a9
LOC100041932	mtDNA_ND2	Rab38	Smyd2
LOC100042199	mtDNA_ND4L	Rab5b	Sord
LOC100044439	mt-Nd4l	Rabgap1l	Sort1
LOC100046770	Myo1c	Ramp1	Sort1
LOC100046853	Napsa	Rapsn	Spag5
LOC100046891	Napsa	Rapsn	St3gal2
LOC100047214	Napsa	Rapsn	St8sia4
LOC100047214	Nav1	Rbbp4	St8sia4
LOC100048123	Ncaph2	Retsat	Stab1
LOC100048461	Ncaph2	Retsat	Stmn1
LOC214403	Ncf2	Rora	Sts
LOC280487	Ncl	Rpl10a	Sulf2
LOC380692	Ndufb11	Rpl18a	Sult1a1
LOC381114	Ngfrap1	Rpl21	Suv39h1
LOC381578	Ngp	Rpl21	Syce2
LOC382707	Niban	Rpl21	Tacc1
LOC384710	Nlgn2	Rpl22	Taf9b
LOC622655	Nme3	Rpl23	Tcea2
LOC633945	Npepl1	Rpl27a	Tgfbr1
LOC640739	Nrcam	Rpl31	Timp2

Cluster 10 Continued
Tk1
Tle1
Tm4sf5
Tm7sf4
Tmem32
Tnfsf12
Tnfsf14
Top2a
Tpp1
Trem2
Trf
Trip12
Trmt1
Usp28
Usp7
Vegfb
Vkorc1
Vkorc1
Vkorc1
Wdr77
Wtip
Zcchc14
Zdhhc3
Zdhhc8
Zfp367
Zfp496
Zkscan17

Cluster 11			
1110059P08Rik	BC030335	Eif4a1	Insig1
1200002N14Rik	BC039210	Eif4e	Irak2
1700047I17Rik1	Bcl6	Eif4g1	Itga5
1700047I17Rik1	Bdh2	Eif4g1	Itgav
1700108L22Rik	Brd2	Eif4g2	Itgb4bp
2210412D01Rik	Btaf1	Eif6	Jag1
2310047D13Rik	Bzw1	Elavl1	Kcnn4
2310061F22Rik	C630022N07Rik	Ell2	Kif1b
2410042D21Rik	Camk2d	Elovl1	Kif1b
2510009E07Rik	Ccnc	Emilin2	Kif1c
2610028A01Rik	Cct8	Emilin2	Kpna1
2610103J23Rik	Cenpt	Eno2	Krit1
4733401O04Rik	Chic2	Ergic1	Larp-pending
4921505C17Rik	Chic2	Ext1	Lcp2
4933424B01Rik	Chst11	Fbs1	Ldlr
4933428A15Rik	Ciapin1	Flnb	Leng1
6330409N04Rik	Clec2d	Flnb	LOC100046025
6330409N04Rik	Clec4d	Fmnl2	LOC100046855
6330548G22Rik	Clec5a	Fnbp1l	LOC100047393
6330569M22Rik	Clic4	Fyb	LOC100047934
8030448M07Rik	Clic4	Fzd7	LOC271490
A530088I07Rik	Col18a1	Galnt6	LOC433261
A730020L20Rik	Cpeb4	Ggta1	LOC670044
A730042J05Rik	Creb1	Gls	Lrp8
A730098D12Rik	Csf2rb	Gmeb2	Lrrc59
A930029N17Rik	Cts6	Gnb4	Lrrc59
Abce1	Cul1	Gnl3	Lrrc59
Abi1	Cxcl16	Gnptab	Lrrfip2
Abr	Cxcl16	Gpr137b	Lrrfip2
Acbd3	Daam1	Gpr68	Ltv1
Adarb1	Dcp1a	Gpr84	Mafg
Adora2b	Dcun1d3	Gpr84	Mak16
Afg3l2	Ddhd1	Gpr85	Map4k3
Agpat4	Ddx21	Gpr85	Mapk11
Akna	Denr	Gspt1	Mapre1
Alg11	Denr	Gtf2f2	Mark2
Ampd3	Dhps	Gtf3c4	Mast2
Apol7c	Dhx15	Hmgcr	Mast2
Appbp1	Dhx15	Hnrnpab	Mdn1
Arl8a	Dock10	Hsd17b12	Memo1
Atp6v1e1	Dot1l	Hspbap1	Mon1b
Atp6v1h	Dtwd1	Htra1	Mrpl52
Atpbd3	Dus2l	Htra1	Mrps18b
Baz1a	E030040G24Rik	Ifnar2	Mrps18b
BC004044	Eea1	Igf2r	Mtdh
BC004044	Eef1e1	Ikzf1	Mtpn
BC027231	EG627624	Inpp5b	Nck1

Cluster 11 Continued

Nfkb2	Samsn1	Ube2f
Nfyb	Sdc1	Ube2z
Ninj1	Sec24b	Uso1
Nip7	Seh1l	Usp14
Nol10	Seh1l	Usp16
Nol5	Sertad1	Usp2
Nrbf2	Sestd1	Utp11l
Nup62	Sf1	Vasp
Otud4	Sfrs2	Wbp4
Pak4	Sfrs2	Wdr59
Pdcd10	Sfrs3	Wdsof1
Pdlim7	Sfrs3	Ywhae
Peli3	Sh3bgrl	Ywhag
Pgls	Sh3bgrl2	Zc3h7a
Pgs1	Sh3bgrl2	Zc3h7a
Plxna2	Slc30a6	Zfpn1a1
Pou2f2	Slc30a7	Zxdc
Ppfia1	Slc39a13	
Ppfia1	Slc39a13	
Ppfia1	Smad2	
Ppm1b	Smc6	
Ppm1b	Snap29	
Ppp2ca	Spred1	
Ppp4r1	Srfbp1	
Ppp4r2	Srxn1	
Pprc1	St3gal1	
Psd4	St7	
Ptpn11	Syncrip	
Ptpn12	Tbrg4	
Ptpn2	Tcerg1	
Rab10	Tes	
Rab32	Tes	
Rabep1	Tgm2	
Rabep1	Timp1	
Rai12	Tmem168	
Rai14	Tmem38b	
Ranbp1	Tmem38b	
Rapgef1	Tnip2	
Rasa2	Tpm4	
Rasa2	Tra2a	
Rbpms	Trappc10	
Rbpms	Ttc19	
Rexo4	Txnrd1	
Rgs16	U2af2	
Rps6ka4	Uba5	
S100pbp	Ube2d3	
Samd4b	Ube2f	

Cluster 12		
1200016E24Rik	Dnaja2	Rnf220
1520402A20Rik	Dnaja2	Sertad2
2010002N04Rik	Dscr1	Sertad2
2210037E17Rik	Dusp16	Siah2
2310024N18Rik	Dusp16	Skil
2610103N14Rik	Dusp16	Slc20a1
4732435K05Rik	E130014J05Rik	Spsb1
4833438C02Rik	E330037I15Rik	Srebf2
4933417E08Rik	EG622976	St6galnac4
5430414B19Rik	Erdr1	St6galnac4
5730552E08Rik	Frap1	Syncrip
8030462N17Rik	Gna13	Tiparp
9430049I24Rik	Hnrnpd	Tle3
A630072M18Rik	Hnrpll	Tlr2
A630097K09Rik	Il17ra	Tmem23
AI504432	Il1r2	Tnfaip2
Arap1	Il20rb	Trps1
Arap1	Itgp	Ugcg
Arg2	Jak1	Vti1a
Arhgap26	LOC380629	Wsb1
Arhgef3	LOC381140	Zfp382
Auh	LOC432554	Znrf1
B230343A10Rik	Lrp12	
B230378H13Rik	Map3k6	
B830007D08Rik	Map3k7ip2	
Bcl10	Map4k4	
Bhlhb2	Mapk6	
Btg1	Mapk6	
Btg1	Mobkl2b	
C330006D17Rik	Mt2	
C330023M02Rik	Mtf1	
C430002D13Rik	Nfkbib	
C530027B15Rik	Nsbp1	
Cd14	Nsun2	
Cd33	P2ry2	
Cd33	Pde4b	
Cd83	Phf19	
Chd7	Plaur	
Chic2	Plek	
Cias1	Ppp1r10	
Cish	Prdm2	
Crk	Prdm2	
Crk	Rangap1	
Cul1	Rbpms	
D030041G07Rik	Rffl	
D230037D09Rik	Rffl	
D630023B12Rik	Rnd3	

Cluster 13

0610007C21Rik	2410015N17Rik	A430106D13Rik	Arsg
0610007C21Rik	2410018G20Rik	A630097D09Rik	Ascc1
0610007C21Rik	2410018G20Rik	A930005H10Rik	Ascc1
0610009L18Rik	2610014I16Rik	A930010I20Rik	Asph
0610011F06Rik	2610024M03Rik	Abca9	Asxl1
0610037D15Rik	2610029K11Rik	Abcb8	Atg2a
0610040B10Rik	2610036L11Rik	Abcg1	Atg2a
0910001A06Rik	2610039C10Rik	Abhd14b	Atp6v1g2
1110001A07Rik	2610307O08Rik	Abtb1	Atxn1
1110002B05Rik	2610510J17Rik	Acaa2	Aurkb
1110003E01Rik	2610528E23Rik	Acaa2	AV249152
1110012D08Rik	2700033B16Rik	Acaa2	Avpi1
1110013L07Rik	2700045P11Rik	Acaa2	AW112037
1110029I05Rik	2700094K13Rik	Acadm	AW212394
1110032E23Rik	2700094K13Rik	Acads	B930037P14Rik
1110046J11Rik	2810017I02Rik	Acap3	B930041F14Rik
1110062M06Rik	2810410A03Rik	Acot1	Bbs2
1190002A17Rik	2810422J05Rik	Acox3	Bbs9
1300010F03Rik	2900024C23Rik	Acox3	BC018371
1500041B16Rik	3000004C01Rik	Acp2	BC021381
1700023B02Rik	3110001A13Rik	Acpl2	BC023744
1700025G04Rik	3110001O07Rik	Acvr2b	BC024659
1700025K23Rik	3110001P07Rik	Adcy7	BC031353
1700034H14Rik	3830612M24	Add3	BC038156
1700054N08Rik	4632428N05Rik	Add3	Bckdha
1700081H05Rik	4833420G11Rik	Agap1	Bhlhb9
1700109H08Rik	4930402E16Rik	Agxt2l2	Btbd11
1810013B01Rik	4930481A15Rik	AI480653	Btbd6
1810015A11Rik	4930563B10Rik	AI662250	Btbd6
1810044D09Rik	4930577N17Rik	Akr7a5	Bub1b
2010005O13Rik	4933427G23Rik	Aktip	Bub1b
2010011I20Rik	4933437K13Rik	Ank	C430004E15Rik
2010107H07Rik	4933439C10Rik	Antxr2	C730029F17Rik
2210010L05Rik	5031439G07Rik	Anxa9	C79407
2210010N04Rik	5133400G04Rik	Ap1g2	C920007D24Rik
2310005P05Rik	5330438D12Rik	Ap2a2	Calcoco1
2310014D11Rik	5430406J06Rik	Ard1	Camk2b
2310014G06Rik	5730405I09Rik	Ard1a	Casp6
2310040A07Rik	5730550L01Rik	Arhgap15	Ccbl1
2310047K21Rik	6330414G02Rik	Arhgap15	Ccbl1
2310051N18Rik	6430590I03Rik	Arhgap18	Ccdc5
2310061C15Rik	9030607L17Rik	Arhgap6	Ccl27
2310061J03Rik	9130019O22Rik	Arhgef4	Cd163
2410002F23Rik	9330186A19Rik	Arhgef6	Cd209f
2410002F23Rik	9530058B02Rik	Arhgef6	Cd28
2410006H16Rik	9530058B02Rik	Arl4c	Cd300a
2410008K03Rik	9830001H06Rik	Arl4c	Cd3eap

Cluster 13 Continued

Cd79b	Ctnnd2	Entpd4	Gstz1
Cd79b	Ctps2	Eps8	Gtse1
Cd8b	Cxcr3	Espl1	Guk1
Cd97	Cxcr4	F630025I20Rik	Guk1
Cd97	Cxx1a	F730031O20Rik	Gusb
Cd97	Cxx1c	Fads1	Gyg
Cdc14b	Cyhr1	Fahd2a	Gzmd
Cdc20	Cyhr1	Fam102a	H13
Cdc23	Cytip	Fam134b	H2afv
Cdc2a	D030029J20Rik	Fancm	H2afv
Cdc42ep3	D14Ertd449e	Fbxl10	Hddc2
Cdca2	D17H6S56E-5	Fbxl10	Hddc2
Cdca3	D230007K08Rik	Fbxo44	Hectd3
Cdca7	D2Ertd750e	Fcrl1	Helz
Cdca8	D4Wsu114e	Fcrl1	Hfe
Cdca8	D630004K10Rik	Fgfr1op	Hfe
Cdkn2c	D930015E06Rik	Fh1	Hgf
Cdkn2c	D930028F11Rik	Fmo5	Hint2
Cenph	Daglb	Fnbp1	Hist1h1c
Cenpk	Dcun1d4	Foxm1	Hist1h1c
Cenpm	Ddah2	Foxo1	Hist1h2ad
Cenpp	Depdc6	Fvt1	Hist1h2ad
Centd1	Dhrs3	Galnt12	Hist1h2af
Cep55	Dhrs7	Gamt	Hist1h2ah
Cercam	Dhrs7	Ganc	Hist1h2ah
Chn2	Dlgap5	Ganc	Hist1h2ai
Chst3	Dnajc12	Ganc	Hist1h2ak
Chst3	Dph3	Gcc2	Hist1h2an
Cirbp	Dtd1	Ggnbp1	Hist1h2ao
Ckb	Dusp22	Git2	Hist1h3e
Cln6	Dync2li1	Glrx2	Hist1h3f
Clspn	E030020A01Rik	Glt8d3	Hist2h2be
Clspn	E2f2	Glul	Hpgd
Cmtm8	E2f2	Gm114	Hrsp12
Cnksr3	E2f2	Gmpr	Hrsp12
Cnnm2	E330036I19Rik	Gnb5	Hs6st1
Cnnm3	Ebi2	Gnb5	Hscb
Cnrip1	Ebpl	Gpld1	Hsd17b11
Cry2	Ebpl	Gpr155	Ica1
Crygn	Eef2k	Gpr155	Icam2
Cryl1	Efcab4a	Gpr171	Idh2
Cryz	Efcab4a	Gpr19	Ier5l
Cryz	EG328314	Gpr34	Ifngr1
Csad	EG433229	Gpr34	Ift140
Csnk1g2	Eif4ebp2	Gpsm1	Ift140
Ctdsp2	Enc1	Gspt2	Ihpk1
Ctdspl	Entpd1	Gstt3	Il6ra

Cluster 13 Continued

Imp3	LOC100045877	Mras	Pmf1
Impa2	LOC100047226	Mrps31	Pmm1
Iqgap3	LOC100047634	Mxd4	Pnpla7
Itfg3	LOC100047670	Mxd4	Pnpo
Itga6	LOC100047936	Mxi1	Pold4
Itgb5	LOC100048434	Myo5a	Porcn
Ivd	LOC100048845	Myom1	Ppa2
Ivns1abp	LOC100048845	Nacc2	Ppap2c
Ivns1abp	LOC226017	Naprt1	Ppm1d
Ivns1abp	LOC329416	Nav1	Ppp1r9a
Kctd17	LOC333331	Nav1	Prcp
Kif11	LOC380927	Ncapd2	Prep
Kif13a	LOC381629	Ndc80	Prkacb
Kif15	LOC630146	Ndrg3	Prkrir
Kif18a	LOC634327	Ndst1	Prps2
Kif20b	LOC638935	Ndst1	Ptgs1
Kif21b	LOC674195	Ndst1	Ptgs1
Kif23	Lonrf3	Neurl2	Ptgs1
Kif23	Lpin1	Nipsnap1	Ptpn18
Kif2c	Lrrc40	Nostrin	Pxmp4
Kif3a	Lrrc45	Noxo1	Rab6b
Kif3a	Lrrfip1	Nrm	Rabl4
Kifap3	Luc7l2	Nt5dc2	Rad51
Kifap3	Lyplal1	Oxct1	Ranbp10
Klf2	Man1c1	P2ry1	Rapgef6
Klhl21	Man1c1	Pacs1	Rapgef6
Klhl24	Mansc1	Padi2	Rarres2
Klhl6	Map4k1	Paics	Rasgrp3
Klhl6	Map4k2	Paip2	Rbm38
Kmo	Marveld1	Paip2b	Rbm38
Lama3	Mbp	Parp1	Rcbtb2
Leprel2	Mbtps1	Pbk	Rdm1
Lgals4	Mcee	Pcbp4	Rell1
Lig1	Mcm4	Pdcd4	Retnlg
Lig1	Mcm7	Pdcd4	Rgs10
Lman2	Mctp1	Pdcd4	Rhbdd3
Lmnb1	Mdh1	Pde1c	Rhebl1
LOC100039496	Metrn	Pdgfc	Rhoj
LOC100040353	Mfng	Peg13	Rmnd5b
LOC100042970	Mfsd3	Peli2	Rnase4
LOC100043308	Mical1	Pfas	Rnase6
LOC100043821	Mical1	Phactr2	Rnf167
LOC100043821	Mical1	Phka2	Rogdi
LOC100044204	Mkrn1	Pkib	Rom1
LOC100045005	Mospd1	Plscr4	rp9
LOC100045457	Mospd3	Plxnc1	rp9
LOC100045738	Mospd3	Plxnc1	Rrm1

Cluster 13 Continued

Rsrc1	Tbc1d2	Trp53inp1
Samd4	Tbc1d2	Tspan4
Scarf2	Tbc1d5	Ttc3
Scotin	Tbc1d8	Ttc3
Selenbp2	Tbl1xr1	Ttc3
Sema6b	Tbrg1	Ttc7
Sept6	Tcea2	Txnrd2
Sesn1	Tcea3	Ube2h
Sesn1	Tceal1	Uhrf1
Setd7	Tceal1	Ulk1
Setx	Tcf19	Ulk2
Sft2d2	Tex261	Ulk2
Sft2d3	Tfdp2	Unc119b
Sgol2	Tgfbr2	Urod
Sgpp1	Tgfbr2	Vapb
Sidt2	Thbd	Vps13b
Ski	Thtpa	Vrk1
Slc13a2	Tle1	Wbp5
Slc15a2	Tlr5	Wbscr27
Slc15a2	Tmc6	Wdr45
Slc17a5	Tmc6	Wdr51b
Slc19a1	Tmc6	Wdr51b
Slc25a13	Tmem110	Wdr6
Slc39a12	Tmem141	Wipi2
Slc46a3	Tmem141	Wwp1
Slc7a4	Tmem154	Wwp1
Slco2b1	Tmem168	Wwp1
Smad5	Tmem184c	Xpr1
Smap2	Tmem25	Xpr1
Smc3	Tmem53	Ydjc
Smc4	Tmem71	Ypel3
Snhg10	Tmem82	Zdhhc3
Sorl1	Tnfsf13b	Zfhx3
Spc25	Tnfsf13b	Zfhx3
Srgap3	Tnni2	Zfp219
Srpk3	Tnrc6b	Zfp219
Ssbp3	Tns1	Zfp282
St6gal1	Tpcn1	Zfp36l1
Stab1	Tpcn1	Zfp524
Stab2	Tpcn1	Zfp704
Stxbp5	Tpk1	Zfp770
Suclg2	Tpk1	Zfp99
Suclg2	Treml1	Zmat3
Sufu	Trim47	Znf512b
Syp	Trim59	Zzz3
Tardbp	Trim59	
Tbc1d17	Troap	

Cluster 14			
1500011B03Rik	Cnp	Oas1c	Tnnc1
1500019C06Rik	Coq5	Oas1d	Tubg1
1700112E06Rik	Cox6a2	Ocel1	Unc13d
1700113I22Rik	Creb3l1	Ociad2	Unc13d
1700113I22Rik	Cryba4	Ogfrl1	Usp45
2210012G02Rik	Cryzl1	P2ry14	
381484	Cutc	P2ry14	
4930486L24Rik	Cutc	P2ry14	
4930539E08Rik	D14Ertd231e	Pcnp	
4933403G14Rik	D330050I23Rik	Pde7b	
4933425L03Rik	Ddit3	Per2	
5330420J21Rik	Ddit3	Pigl	
5330431N19Rik	Fam100b	Pla2g2e	
5730403B10Rik	Fbxw7	Plekhm1	
6330442E10Rik	Fgfbp3	Pnpla1	
6330442E10Rik	Gm1276	Prei4	
6530401N04Rik	Gng11	Prelid2	
9330175B10Rik	Gpatch2	Ptcd2	
9530048O09Rik	Gpkow	Pten	
Acbd4	Gtf2h2	Ralgps2	
Acbd4	Gypc	Rgs14	
Acpp	Hap1	Rhbdd1	
Adck1	Hist3h2a	Rhov	
Amigo3	Homer1	Rnf141	
Angpt2	Ints9	Rnf141	
Ankle2	Itgam	Samhd1	
Ankle2	Itpr1	Sass6	
Arhgap4	Itprip	Sfxn5	
Arid3b	LOC100036521	Sh3bp1	
Bbs4	LOC100041137	Siglec1	
Bcdo2	LOC100045266	Sirpb1	
C330008K14Rik	LOC100046843	Sirpb1	
C79267	LOC100047419	Slc12a9	
C79267	LOC381524	Slc16a6	
Capn10	LOC386288	Slc2a9	
Casp3	Lrrk2	Stoml1	
Ccdc125	Ltb4r1	Stoml1	
Ccdc134	Mag	Stoml1	
Ccdc99	Ms4a6b	Tbl2	
Ccl25	Msl3l2	Tbxas1	
Ccl25	Mtmr3	Thada	
Cd1d1	Nat6	Thbs1	
Cd22	Nisch	Timeless	
Cd300lf	Nsmaf	Tlr13	
Cdk5rap2	Nsun4	Tmcc3	
Cep164	Nt5m	Tmem126b	
Cln8	Nt5m	Tnfrsf26	

Cluster 15	
1500041J02Rik	Spata3
3010031K01Rik	Vegfa
5430434G16Rik	Wdr62
8030474H12Rik	Zfp703
8430403J19Rik	
A430073A17Rik	
Areg	
Chka	
Cited2	
Cited2	
Cited2	
Cpeb2	
Csf2	
Dpep3	
Dusp1	
Egr1	
Egr2	
Egr3	
Egr4	
Errfi1	
Fos	
Fosb	
Fosl2	
Gem	
Grasp	
Id3	
Idb2	
Ier2	
Ier3	
Irs2	
Jmjd3	
Jun	
Klf4	
Lcp2	
LOC100045678	
LOC240672	
Mapk6	
Myc	
Nfkbid	
Osm	
Pde4b	
Pim3	
Plau	
Pmaip1	
Pmaip1	
Sfrs5	
Smad5	

Cluster 16

1500001E21Rik	Cd300lb	Fhod1	Mras
1700034H14Rik	Cdc45l	Fibp	Mrps25
1700034H14Rik	Cebpa	Fignl1	Mrps25
1700123D08Rik	Cenpi	Fmnl1	Mthfd1
2310022K01Rik	Chd6	Fuca1	Mybl2
2310038D14Rik	Chtf18	Fvt1	Nanos1
2610002D18Rik	Ckap2	Galnt9	Nap1l1
2610020C11Rik	Cmbl	Gart	Nap1l1
2610203C22Rik	Col4a1	Gbl	Ncf2
2610302F08Rik	Col4a5	Gbl	Ncoa4
2810405K07Rik	Col4a5	Gbl	Nrp
2810408P10Rik	Crem	Gemin4	Nuak1
3830430K15Rik	Crem	Gins1	Nup85
4932441P04Rik	Cspp1	Gins2	Olfml3
5730407K14Rik	Cx3cr1	Gmnn	OTTMUSG00000000421
5930418K15Rik	Cxcl14	Hey1	Pcgf6
6330403M23Rik	Cxcl14	Hirip3	Phka2
7530408C15Rik	Cyp2s1	Hist1h3f	Pla2g15
9330147J08Rik	D030063E12	Hprt1	Pole
9530053J19Rik	D230014K01Rik	Idh1	Polr1b
9630025I21Rik	D630036H09Rik	Idh3a	Polrmt
9830144J08Rik	D6Wsu163e	Il27ra	Pparg
Acot2	Dm15	Kntc1	Pparg
Adal	Dmkn	Lbcl1	Ppm1l
Adssl1	Dmkn	Lbr	Prmt7
Adssl1	Dnajc28	Lmnb2	Psmc3ip
Ahrr	Dtymk	LOC100041567	Psmf1
Angptl4	Dtymk	LOC100045677	Ptchd1
Antxr2	E030038D23Rik	LOC100047264	Ptger2
Arhgef1	E130016E03Rik	LOC100047273	Rad50
Arrb1	E230029C05Rik	LOC100048280	Rad51
Arrb1	E230029F23Rik	LOC237877	Rad51ap1
Asf1b	E330009F12Rik	LOC382264	Rad51ap1
Atad2	E430018J23Rik	LOC384453	Rgs11
AU020206	Ear2	LOC385659	Rnf167
AV028368	Ear2	Lrrc39	Rnf167
B230312I18Rik	Ear3	Map2k6	Rnmt
B930008G03Rik	EG226654	Mcm10	Rnmt
Blm	Eif4a2	Mcm10	Rpa2
Brip1	Ermp1	Mcm3	Rpa2
Brp16	Esco2	Mcm5	Rrm2
C85492	Exosc6	Mcm6	Serpinb6b
Camk2n2	F630043A04Rik	Mcm6	Serpine2
Cand1	F730047E07Rik	Mcm6	Sesn1
Ccne2	Fancd2	Mcm7	Sgol1
Cd151	Fanci	Melk	Shcbp1
Cd207	Fen1	Mphosph9	Slc24a3

Cluster 16 Continued
Slc27a3
Slc37a1
Slc37a2
Sort1
Sox4
Spc25
Tbx6
Tdrd3
Tfrc
Tfrc
Tgfb3
Tk1
Tmem48
Tmem81
Trub1
Trub1
Tspan33
Ttbk2
Tyms
Tyms
Tyms-ps
Ube2t
Ung
Vamp5
Wdr90
Wrb
Wwp1
X99384
Zfp101
Zfp119
Zfp60
Zfp608
Zfp760
Zfp87
Zfp87
Zfp94

Cluster 17

0610006I08Rik	A530055J02Rik	Brrn1	Decr1
0610006I08Rik	A630006E02Rik	C030048B08Rik	Dedd2
0910001L09Rik	A630091F01Rik	C130022E19Rik	Dfna5h
1110018J18Rik	Aaas	C330006A16Rik	Dhrs7
1110018J18Rik	Abcc3	C76746	Dnahc11
1110049F12Rik	Abhd11	C79407	Dnajc12
1500010J02Rik	Abhd12	Cacna1f	Dppa3
1500010J02Rik	Abhd8	Camk1	Dscr1l2
1500011H22Rik	Accs	Camk1	Dym
1700008F19Rik	Acp6	Camk1	Dyrk1b
1700026L06Rik	Acss1	Camk1d	E230008N13Rik
1700041B20Rik	Actr8	Camk2g	Ech1
1810009O10Rik	Adi1	Car9	Echs1
1810020D17Rik	Adipor1	Car9	EG546143
1810030N24Rik	Adssl1	Casp6	EG623230
2210011C24Rik	Akap8l	Ccdc120	EG667410
2210404O07Rik	Akr7a5	Ccdc28a	Ela1
2210410E06Rik	Akr7a5	Ccrk	ENSMUSG00000053178
2310005E10Rik	Aldh4a1	Cdan1	Etaa1
2310005L22Rik	Aldh4a1	Cdc14b	Ethe1
2310007H09Rik	Alg6	Cdk5rap3	Eya1
2310007H09Rik	Als2cr13	Ceacam2	Fahd1
2310047A01Rik	Als2cr13	Cenpq	Fam108a
2310047B19Rik	Ankmy2	Chaf1a	Fam110a
2310067B10Rik	Anks6	Chst14	Fam110a
2410091C18Rik	Ap1b1	Cln6	Fam122b
2610003J06Rik	Appl2	Cluap1	Fam168a
2610044O15Rik	Arhgap19	Cnot6l	Fam168a
2700084L06Rik	Arhgap19	Comt	Fbxl12
4833420G17Rik	Arhgap24	Comt	Fbxl20
4833427B12Rik	Arhgap9	Ctla2b	Fbxo21
4930534B04Rik	Armc3	Ctnnbip1	Fbxw4
4930572J05Rik	Armc3	Cugbp2	Fchsd1
4931406C07Rik	Arrdc2	Cugbp2	Fgd2
4933427D14Rik	Asnsd1	Cxx1b	Fhod1
5033430I15Rik	Atp13a2	Cyb5r1	Gab3
5430405C01Rik	AW544981	D10627	Galt
5730403M16Rik	B130019G13Rik	D10Ertd322e	Galt
5730455P16Rik	B230342M21Rik	D330008I21Rik	Gats
9030025P20Rik	B3gnt1	D3Ertd300e	Gats
9030025P20Rik	B3gnt8	D6Wsu176e	Gcdh
9030025P20Rik	B3gnt8	D6Wsu176e	Gcnt1
9130211I03Rik	Bag4	D930015E06Rik	Gcnt1
9130221H12Rik	BB128963	D930049D19Rik	Gdpd3
9530064J02	BC038925	Dalrd3	Gdpd5
A230057M07Rik	BC039093	Dbndd2	Ggcx
A530050E01Rik	Bin1	Dbp	Gigyf1

Cluster 17 Continued

Gm114	Klc4	Naprt1	Pou6f1
Gm166	Klhdc2	Nat15	Ppapdc2
Gna12	Ldb1	Ncapd3	Ppp1r12c
Gng2	Lincr	Ncapg2	Prkcb
Gnpda1	Lincr	Ncapg2	Prkra
Gnpda1	Lman2l	Ncaph	Prr12
Gnpda1	Lnx2	Ndufb8	Prune
Golm1	LOC100040799	Nfatc1	Prune
Golm1	LOC100044159	Nfic	Ptplad2
Gpn3	LOC100044204	Nfic	Ptpn22
Gpr146	LOC100047028	Nhlrc1	Pus3
Gpr146	LOC100047260	Nicn1	Pycrl
Gpr162	LOC100047762	Nme4	Pygb
Gpr162	LOC100047863	Nme4	Pygl
Gpt1	LOC100048589	Nme7	Rab11fip5
Gtf3a	LOC269515	Nosip	Rab11fip5
Hagh	LOC381691	Npal3	Rab3il1
Haghl	LOC382010	Nqo2	Rab4a
Haghl	LOC383077	Nr2f6	Rabl2a
Havcr2	LOC628836	Nudt19	Rap1gap
Havcr2	LOC668047	Nudt7	Rasgrp4
Hdac10	LOC674427	Nudt7	Rassf2
Hdac5	LOC676420	Ogfod2	Rassf3
Hdac5	Lrmp	Oit3	Rassf5
Hebp1	Lrmp	Ophn1	Rb1
Hemk1	Lrrc27	Osbpl11	Rb1
Hes6	Lrrc28	P2rx7	Rbak
Hexdc	Lyl1	P2rx7	Rcan3
Hip1	Mad1l1	P2ry12	Rcbtb2
Hisppd2a	Manba	Pacrg	Rev3l
Hist1h2bm	Manba	Pcnt	Rfc4
Hmg20a	Map3k1	Pcyox1	Rfx1
Hmgcl	Map3k14	Phf3	Rfxap
Hmgcl	Mat2b	Pias3	Rhobtb1
Hs2st1	Mcm2	Pias3	Rhobtb1
Hyi	Mfsd7b	Pigyl	Rnf150
Ift122	Mgst2	Pigz	Rnf167
Ift81	Mknk2	Pkib	Rpa1
Il6ra	Mospd1	Pkib	Rpa3
Impa2	Mospd1	Pkig	Rrm1
Iqce	Mpg	Pkig	Rrp1b
Iqgap2	Mpst	Plcb2	Rsdr1-pending
Iqgap2	Msh6	Plekhg3	Sac3d1
Irf2bp2	Msh6	Plxdc1	Sars
Itgb3bp	Mta3	Pmm1	Scamp5
Jarid1b	Mtr	Poli	Scp2
Kdelc1	Myo18a	Polk	Sema4b

Cluster 17 Continued

Sepn1	Tpmt
Setd2	Tpra40
Sgsh	Trmt2b
Sh3bp5l	Trove2
Sirt3	Tspan32
Sirt5	Ttc39a
Slc16a6	Ttc7
Slc2a3	Uchl3
Slc2a8	Uhrf1bp1l
Slc35c2	Umps
Slc41a3	Usp30
Slc46a1	Vps33b
Smarca2	Wbp1
Smg6	Wbp1
Smpd2	Wdr21
Snta1	Wdr81
Snx14	Wipi1
Snx33	Wwp1
Snx5	Xrcc1
Snx5	Xrcc6
Spint1	Xrcc6
Spr	Yipf2
Ssbp3	Zc4h2
Susd3	Zdhhc14
Taf3	Zer1
Tatdn3	Zfhx2
Tbxas1	Zfp202
Tbxas1	Zfp251
Tctn3	Zfp280c
Tctn3	Zfp36l1
Tec	Zfp36l1
Terf2	Zfp414
Them2	Zfp707
Tipin	
Tk2	
Tle6	
Tm6sf1	
Tm7sf3	
Tmem107	
Tmem143	
Tmem65	
Tmem8	
Tmem8	
Tmem9	
Tnfrsf22	
Tob1	
Tpmt	

Cluster 18

0610009B22Rik	3010026O09Rik	A530017D24Rik	B930030B22Rik
0710007G10Rik	3110078M01Rik	A530082C11Rik	Bace1
1110001C23Rik	4632417N05Rik	A630084D02Rik	Bbc3
1110003O08Rik	4732486I23Rik	A730063M14Rik	Bbc3
1110008L16Rik	4733401I05Rik	A730063M14Rik	BC002230
1110032A13Rik	4831403C07Rik	A930008G19Rik	BC003498
1110032A13Rik	4832420L08Rik	A930026I22Rik	BC019806
1110034G24Rik	4833442J19Rik	AA415398	BC025076
1110048D14Rik	4930429O20Rik	Aasdh	BC049806
1110058L19Rik	4931426K16Rik	Aatk	BC053749
1110068E08Rik	4932417I16Rik	Abcb7	Bckdk
1190002N15Rik	4933411K20Rik	Abcc10	Bcl2l13
1190028F09	4933439C20Rik	Abcd2	Bcl7a
1200011O22Rik	5330401P04Rik	Abcd2	Bcl9l
1300007F04Rik	5330401P04Rik	Acn9	Bcs1l
1500004F05Rik	5330414O08Rik	Acn9	Best1
1500012M23Rik	5630401D24Rik	Acot1	Bmf
1700012H17Rik	5630401D24Rik	Acsf3	Brwd1
1700021K19Rik	5730419I09Rik	Acsl3	Btbd1
1700023B02Rik	5730593F17Rik	Adal	C030006K11Rik
1700023B02Rik	5730601F06Rik	Adcy7	C030010B13Rik
1700027M01Rik	5730601F06Rik	Adcy9	C030014I23Rik
1700027M01Rik	5830454E08Rik	Adrb2	C030025P15Rik
1700034H14Rik	6030413G23Rik	AI595366	C530044N13Rik
1700052N19Rik	6230416J20Rik	AI847670	C730026J16
1700065O13Rik	6230427J02Rik	AI875142	C87436
1810007P19Rik	6330403E01Rik	Als2cr13	C920006O11Rik
1810011H11Rik	6330419J24Rik	Ankrd10	Cables1
1810011H11Rik	6430511F03	Ankrd47	Casp2
1810049H13Rik	6430540M20Rik	Anks6	Cbr3
1810063B05Rik	6430548M08Rik	Ap4m1	Cbx6
2010004M13Rik	6430548M08Rik	Ap4m1	Cbx7
2010312A17Rik	6530415H11Rik	Ap4m1	Cbx8
2010320M18Rik	6720456H20Rik	Aph1c	Ccdc85b
2210408F11Rik	9130204C11Rik	Aplf	Cdc25b
2210419D22Rik	9230112E08Rik	Arl11	Cdc6
2310035K24Rik	9330133O14Rik	Arrb1	Cdk5rap1
2310036D04Rik	9330175B01Rik	Asb2	Cenpm
2310036D22Rik	9430093I07Rik	Atg2b	Cenpn
2310040G24Rik	9530055J05Rik	Atmin	Clspn
2610035D17Rik	9630015D15Rik	Atp10d	Cmkbr2
2700049A03Rik	9930012K11Rik	Atp5sl	Cnot10
2810410C14Rik	A130010J15Rik	Azi1	Cnr2
2810440J20Rik	A130039H17Rik	B3gnt9	Cnr2
2900024O10Rik	A130092J06Rik	B3gnt9	Comt
2900026A02Rik	A230106J09Rik	B430320J11Rik	Coq9
2900057K09Rik	A430107D22Rik	B4galt4	Cryz

Cluster 18 Continued

D15Bwg0759e	Fut10	Kifc1	Msrb2
D630014A15Rik	Fut10	Klhdc5	Mtus1
D6Wsu163e	Galnt10	Klhl17	Mtus1
D830050J10Rik	Gm561	Klhl24	Myst4
D930048N14Rik	Gm561	Klhl6	Nat8l
Ddx26b	Gne	Krba1	Nckipsd
Deb1	Gpr160	Ldoc1l	Ndor1
Def6	Gpr65	LOC100045780	Ndor1
Dgkd	Gpsm1	LOC100046254	Nedd9
Dgkg	Gstt2	LOC100046775	Nedd9
Dhcr7	Gtf2i	LOC100047480	Nek3
Diras2	Gtf2ird2	LOC100047839	Neurl
Diras2	Gtf2ird2	LOC225897	Nfam1
Dnajc28	Gtf3a	LOC270152	Nfatc1
Dnase2a	Gyg	LOC384890	Nisch
Dph3	H1f0	LOC386486	Nmnat3
E030007N04Rik	H2afj	LOC670089	Notch4
E130102H24Rik	H2afj	Lpin1	Nrp1
E130302P19Rik	Hes6	Lrig3	Nrp1
E130306D19Rik	Hes6	Lrrc27	Nthl1
E330020G21Rik	Hgf	Lrsam1	Nuak1
Efcab4a	Hist1h3a	Maf	Nudt18
Efnb1	Hist2h2be	Maf	Nudt6
EG545216	Hmgcl	Mafb	Numa1
Elac1	Hmha1	Map3k7ip1	Obfc1
Elmo2	Hoxa7	Mapk14	Olfr802
Elp3	Hoxb4	Mapk1ip1	Orc3l
Eno3	Hscb	Mapk1ip1	ORF19
Epn2	Ick	Marveld1	Otub2
Ercc5	Ick	Mast3	P2ry5
Ercc5	Ift81	Mbd5	Pank1
Erp29	Il16	Mbp	Paqr7
Etl4	Incenp	Mef2c	Parc
F8a	Ing3	Mef2c	Parp2
Fam110b	Ing4	Mef2c	Parp2
Fam119a	Ing4	Megf8	Patz1
Fam160a2	Ing4	Meis1	Pbx2
Fance	Ino80b	Mett11d1	Pcmtd2
Fbxl6	Ints7	Mib2	Pcyox1l
Fbxo10	Irak4	Mknk1	Pcyt1b
Fbxo31	Irf2bp1	Mknk1	Pdcd4
Fbxo32	Itfg2	Mks1	Pdik1l
Fbxo32	Kank3	Mlycd	Pdss2
Fdxr	Kctd12b	Mns1	Pdxk
Foxred2	Kctd12b	Mpg	Pex11c
Frat2	Kctd12b	Msh2	Pex6
Fuk	Kctd2	Msrb2	Pfkfb2

Cluster 18 Continued

Pfkfb4	Rmnd5b	Tmem103	Zfp579
Pfkm	Rnf166	Tmem132a	Zfp692
Pgm2l1	Rnf167	Tmem143	Zfp810
Phf14	Rnu6	Tmem144	Zfp90
Phf7	Rragb	Tmem19	Zfp97
Phlda3	Rttn	Tmem38a	Zfyve27
Phlda3	Rwdd2	Tmem41a	Zkscan14
Pick1	Rxrb	Tmem51	Zkscan5
Pik3c3	Samd10	Tnfaip8l2	Zmym3
Pik3ip1	Sec14l1	Tnfrsf21	
Pik3r2	Sec14l1	Tns4	
Pik3r4	Sec14l1	Top3b	
Pla2g4b	Sec15l1	Triap1	
Plekhg3	Sec22c	Trim62	
Plxdc1	Serac1	Trmt2b	
Pold1	Sirt5	Trove2	
Polg2	Siva1	Trp53inp1	
Polg2	Skp2	Trrp2	
Poll	Slc16a13	Tspan14	
Ppapdc2	Slc16a13	Tspan4	
Ppm1m	Slc19a2	Ttc30b	
Ppp2r5e	Slc25a16	Ttll4	
Prkag2	Slc35a5	Txnip	
Prkd2	Slc35a5	Ulk1	
Pscd1	Slc35c2	Use1	
Pter	Slc37a4	Usp20	
Ptpdc1	Slc40a1	Vars	
Ptpn21	Slc5a6	Vgll4	
Ptpro	Smpd3	Wdr18	
R3hcc1	Snrk	Wdr18	
Rab3a	Snx14	Wipi2	
Rab40c	Snx21	Xpa	
Rad54l	Socs6	Xpc	
Rage	Sp3	Ypel3	
Rapgef6	Spata2L	Zbtb45	
Rasa3	Spry3	Zbtb45	
Rasa3	Ssbp2	Zfhx2	
Rbak	Stard8	Zfp12	
Rchy1	Stard8	Zfp148	
Recql5	Suv420h2	Zfp161	
Rgl2	Synpo	Zfp251	
Rgl2	Taf6	Zfp277	
Rgs18	Tarsl2	Zfp277	
Rgs2	Tef	Zfp383	
Rgs2	Tep1	Zfp39	
Rhod	Thap11	Zfp472	
Ripk1	Tle1	Zfp472	

Cluster 19

1190002H23Rik	Cul2	LOC100040573	Rab12
1190008K20Rik	Cybb	LOC100044376	Rabgef1
1200009I06Rik	Cyld	LOC100045737	Ralgds
2310004N11Rik	D630048P19Rik	LOC100045950	Rcan1
2600010E01Rik	Dnajb6	LOC100048721	Rcl1
2610030H06Rik	Dock10	LOC100048858	Rcl1
2810470D21Rik	Dusp4	LOC666025	Rel
3110043O21Rik	Dusp4	LOC667337	Relb
3830421F03Rik	E2f5	LOC676704	Rffl
4921514E18Rik	Edn1	Lrch1	Rilpl2
4930461P20Rik	Ehd1	Ly9	Ripk2
9030216K14Rik	Eif2c2	Malt1	Rnf12
9030611K07Rik	Eif2c3	MALT-1	Rnf14
9830169E20Rik	Eif5	Manbal	Rnf2
A530045M11	Elavl4	Mapk1ip1l	Rnf38
Aff1	Etf1	Marcks	Rod1
Aff1	F630047D10Rik	Marcksl1	Rps6ka3
Ak2	Fas	Marcksl1	Rusc2
Ankrd50	Fas	Marcksl1	Sbds
Arl5c	Fbxl5	Mdm2	Sdc4
B3gnt5	Fgd6	Mdm2	Sestd1
B4galt5	Flrt3	Mdm2	Sf1
BC003324	Fmn	Mlp	Skil
BC031781	Fndc7	Mmp13	Slc25a25
Bcl2a1b	Gas7	Mmp13	Slc4a7
Bcl2a1c	Gas7	Mtmr14	Slfn2
Bcl2a1d	Gas7	Ndel1	Sntb2
Bcl2a1d	Gdap10	Nfkbie	Snx18
Bcl2l11	Gja1	Nlrp3	Sox12
Bcl3	Gna13	Nup98	Spn
Birc2	Gnal1	Nup98	Sqstm1
Birc3	Gpr109a	Nupl1	Srfbp1
C130032J12Rik	Gramd1a	Orc4	Srgn
C230067O06Rik	Gramd1b	Patl1	Srgn
Casp4	Hbegf	Pcgf3	Ssfa2
Ccdc82	Hipk2	Pi4k2b	St6galnac4
Cdc42ep2	Hnrpdl	Pip5k1a	Stk40
Cdkn1a	Hook3	Pip5k1a	Stx11
Cdkn1a	Ibrdc3	Pkn2	Stx3
Cdkn1a	Icam1	Plagl2	Stx6
Cdkn2b	Il1a	Plscr1	Tacc1
Ch25h	Il1a	Plscr1	Tagap
Clec4e	Il4ra	Ptgs2	Tagap
Csf1	Klf7	Ptpn23	Tagap1
Csnk1d	Klf7	Pvr	Taok1
Csnk1d	Klf7	Pvr	Taok1
Csnk1d	Lmo4	Pxn	Tcp1

Cluster 19 Continued
Tdg
Tlr6
Tmem2
Tmem2
Tmem2
Tmem39a
Tnip1
Tollip
Traf1
Traf2
Traf3ip2
Tsc22d2
Tspyl3
Tubb6
Ube3a
Ugcg
Vps37c
Ybx3
Yrdc
Zc3h12a
Zc3h12a
Zeb2
Zfp131
Zswim4

Cluster 20	
0610010F05Rik	LOC380741
4932409G17Rik	LOC433943
4933439M10Rik	LOC624083
5530401A10Rik	Lrch3
5830407P18Rik	Mllt3
9030612M13Rik	Mllt3
9030619K07Rik	Morf4l1
9930016I07Rik	Msi2
A130019H11Rik	Mtssk
A530020A01Rik	Ncoa6ip
A530089A20Rik	Nfatc3
Acap2	Peli1
Acss2	Pira2
Arhgap10	Pira3
Arrdc4	Pira6
B130008D24Rik	Pira6
B230342N21Rik	Plac8
B430305P08Rik	Ppargc1b
Bach1	Ppp2r3a
BC003314	Slc23a1
BC030336	Stk19
C630011I23	Tbc1d20
Cd164	Tbl1x
Cep110	Vps13c
Chd1	Wins2
Chd1	Wwox
Cnp1	Xrn2
Creb5	Zfp26
D130077B20Rik	Zfp26
D16Ertd472e	Zfp91
D430035B07Rik	Zfp91-cntf
Ddx24	
Dph5	
Gprc5b	
Hspa4l	
Ifi203	
Ifitm6	
Ift172	
Ift172	
Ilf3	
Kctd6	
Kif9	
LOC100038908	
LOC100040462	
LOC100043402	
LOC100046746	
LOC270589	

Cluster 21

1700007P14Rik	Brcc3	Galns	LOC385019
1700020O03Rik	Bst2	Gapdh	LOC385780
1810033B17Rik	C1rl	Gba	LOC547343
1810047K05Rik	C1rl	Gde1	LOC56628
2010005H15Rik	Cadps	Gla	LOC667597
2010305C02Rik	Capg	Glipr2	Lrrc8
2600001G24Rik	Carhsp1	Gp6	Lst1
2810035J02Rik	Casz1	Gpr35	Ly6a
2900090M10Rik	Ccdc23	Grina	Ly6c1
4930578N16Rik	Ccdc41	Gstm2	Ly6e
4930583H14Rik	Cd22	Gstm2	Ly6e
5330431N19Rik	Cd38	Gstt4	Mical2
9030425E11Rik	Cd52	Gtpbp8	Mill2
9130230L23Rik	Cdc37l1	Gtpbp8	Mocs2
9130230L23Rik	Cdkn1c	Gys1	Mov10
A530023O14Rik	Cdkn2d	H2-Q7	Mov10
A530023O14Rik	Cerkl	H2-T22	Mrpl47
A530064D06Rik	Chchd10	H2-T23	Mxd1
A530064D06Rik	Chchd6	H2-T23	Ndufs4
A630012P03Rik	Clic5	Hmga1	Ndufs4
A630082K20Rik	Crcp	Hspa1a	Nes
A630085K21	Csprs	Hspa1b	Nfxl1
AA691260	Ctsk	Hspa1l	Odz4
Abi3	Cyfip2	Hyal1	ORF9
AI427809	Cyln2	I830077J02Rik	Orm1
Aif1	D11Lgp2e	Ifi44	Orm2
Aif1	D130040H23Rik	Il12rb1	Osbp2
Aifm2	D330045A20Rik	Il21r	OTTMUSG00000002038
Akap12	D330045A20Rik	Il21r	Parp11
Akr1a4	Dap3	Inpp4b	Pdk3
Aldh18a1	Dcakd	Insl6	Phospho2
Ankfy1	Dcn	Itgb7	Pkp4
Antxr1	Ddx19b	Kcnab1	Plcl2
Antxr1	Dera	Kcnab1	Ppp2r5c
Ap3m2	Dgkz	Kctd1	Pqlc2
Apol9b	Dnajc2	Klra2	Prm1
Arg1	E030034P13Rik	Lgals3bp	Prm1
Arg1	Eed	LOC100042427	Pstpip1
Armcx6	EG277333	LOC100042427	Ptk2
Ascc2	EG433016	LOC100042427	Pwwp2b
Atf7ip	EG545385	LOC100046746	Rabl5
B130024L21Rik	Elk3	LOC100047052	Rpe
B930075F07	F8	LOC100047904	Scoc
BC017643	F8	LOC223672	Scyb11
BC094916	Fcgr4	LOC331595	Sell
Bcap31	Gadd45g	LOC381048	Serf1
Brcc3	Gadd45g	LOC381787	Siglece

Cluster 21 Continued
Siglece
Siglece
Slamf9
Slc24a6
Slc36a1
Slc39a3
Slc6a12
Slc6a19
Slfn4
Slpi
Smcr7
Smox
Smox
Snx7
Spsb4
Stat1
Taf12
Tbc1d9
Tcf7l2
Tlcd2
Tlcd2
Tmem33
Tmprss4
Tmsb10
Tmsb10
Tpst2
Trib3
Trib3
Trim29
Ufsp1
Ugt1a6a
Ugt1a6b
Upk3b
Vamp4
Vegfc
Wdr20b
Wnk2
Wnt6
Wnt6
Xylt2
Yipf6
Zdhhc24
Zfp821
Znhit1

Cluster 22

0610037L13Rik	BC003885	Eif5	Lrrc59
1100001F19Rik	Bcl2l1	Eif5a	Lrrfip2
1200015F23Rik	Bcl2l1	Elovl1	Mgat4b
1200015F23Rik	Bfar	Epb7.2	Mier3
1700009P03Rik	Birc2	Esd	Mina
1810013L24Rik	Bnip2	Eya3	Minpp1
2010311D03Rik	C230091E03Rik	Fam176b	Mobk1b
2310036I02Rik	Caprin1	Fubp1	Mobkl1a
2310047O13Rik	Cav1	Fusip1	Mobkl2c
2400003C14Rik	Ccdc93	G3bp1	Mrpl3
2410042D21Rik	Ccnc	Gabpb1	Msn
2510010F15Rik	Ccnc	Gabpb1	Mtch2
2810407C02Rik	Ccnyl1	Gfm1	Mtmr14
2810408M09Rik	Cct3	Gnb1	Nab1
2810408M09Rik	Cct3	Golt1b	Nab1
2900097C17Rik	Cct6a	Gosr2	Naif1
4833439L19Rik	Cct6a	Gosr2	Nfkbie
4933401P20Rik	Cd74	Gpc1	Nipsnap3a
5730589K01Rik	Cd86	H2-Ab1	Nol5a
6430527G18Rik	Cdc42se1	H2-Ab1	Nsfl1c
9430065L19Rik	Chuk	Hip2	Nup54
9930111J21Rik	Clic4	Hn1l	Pcdh7
A830007F21Rik	Col18a1	Hnrnph1	Pcmt1
Abcf1	Col4a2	Hnrnph1	Pfkp
Abi1	Crk	Idh3a	Phgdh
Abpb	Crkl	Ikbkg	Pi4k2b
Acsl4	Crlf3	Ilf3	Pias4
Acsl5	Crls1	Ilf3	Pik3ap1
Acsl5	Csda	Irak2	Pkn2
Acvr1b	Csf2rb	Itpkb	Plekha1
Aebp2	Csf3r	Khdrbs1	Pofut2
Agfg1	Ctps	Krit1	Pot1b
Agpat1	Ctps	Larp4	Ppfibp1
AI848100	Cugbp1	Lcp1	Ppfibp2
Ak2	Cul2	Lmnb1	Ppfibp2
Alg8	Cux1	LOC100040505	Ppp2r2a
Anp32a	Cyp20a1	LOC100043675	Ppp3cc
Ap2b1	D11Moh35	LOC100048726	Ppp4r1
Ap2b1	D4Wsu132e	LOC100048796	Psmd10
Apob48r	Dctn4	LOC224732	Psmd5
App	Dhrs9	LOC245350	Psmd7
Arhgap10	Dnajb6	LOC384315	Pstpip2
Arpc5	Dnm1l	LOC432555	Ptafr
Asb6	Dnm1l	LOC432730	Ptbp1
Atg4d	Dnm1l	LOC434200	Ptges3
Atic	Eif3b	LOC629364	Ptk9
BC003885	Eif4a1	LOC630729	Ptpn12

Cluster 22 Continued

Ptpn12	Tagln2
Ptpn12	Tcp1
Ptpn12	Tes
Ptprj	Tlr1
Pvrl2	Tlr6
Rab18	Tnfrsf1a
Rab7	Tnfrsf1a
Rac3	Tnfrsf1a
Rad9	Tnfrsf1b
Raet1b	Tnfrsf1b
Ran	Tomm40
Rars	Tpm3
Raver1	Tpm4
Rg9mtd2	Traf5
Rgs3	Tssc1
Rhoc	Ttc39b
Rhoc	Ube2e1
Rnf11	Ube2f
Rnf111	Ubqln1
Rnf14	Ubxd7
Rnf41	Ubxn8
Rwdd1	Usp12
Sbno1	Usp6nl
Sfrs10	Usp7
Sfrs2	Vcam1
Sgk3	Vdac3
Sgk3	Vps26a
Sgk3	Wapal
Sgk3	Wdr1
Sla	Wdr37
Sla	Wdr37
Slc16a10	Zdhhc6
Slc44a1	Zdhhc6
Slc7a7	Zfp207
Slc7a7	Zfp238
Slc9a8	Zfp238
Spata5l1	
Spcs3	
Spic	
Spic	
Srm	
Srp54	
Srxn1	
Srxn1	
St3gal1	
Stip1	
Stx3	

Cluster 23

0610007L01Rik	Atf4	Dgkh	H2-Ab1
1190003J15Rik	Atf4	Dnmt3l	H60a
1200015F23Rik	Atxn2	Dnmt3l	Hck
1200016B10Rik	Batf	Dst	Heatr6
1500034J01Rik	BC004022	Dtx2	Hivep1
2310047O13Rik	BC006779	Dtx2	Hsp90aa1
2400003C14Rik	BC006779	Dtx2	Ifi205
2400003C14Rik	BC017647	Dync1li1	Ifi205
2410131K14Rik	Bid	E2f3	Ifngr2
2610029J22Rik	Bmp1	E430021P16Rik	Ifngr2
2810408M09Rik	Brd2	Eaf1	Igf2bp2
2810423A18Rik	Brd2	Ebi3	Ikbke
2810474O19Rik	C030046E11Rik	Edg2	Il15
2810474O19Rik	C78339	Edg2	Il15
4632411B12Rik	Camk2d	EG432649	Il15
4930461P20Rik	Camk2d	EG665369	Il1rn
4933426M11Rik	Camk2d	EG665685	Il1rn
5730525O22Rik	Car13	Etv6	Il1rn
5730528L13Rik	Casp4	F10	Il27
6030448M23Rik	Casp4	Fabp3	Il6
6330548G22Rik	Casp7	Fgd6	Irg1
A030007L17Rik	Ccdc50	Fip1l1	Irg1
A030007L17Rik	Ccdc93	Frag1	Itga5
A130015P11Rik	Ccnd2	Fscn1	Itpkb
A630077B13Rik	Ccnd2	Gabpa	Jak2
Acp5	Cd40	Gbe1	Jak2
Acsl4	Cd40	Gbp5	Jmjd6
Acsl4	Cd40	Gca	Katna1
Acsl5	Cd40	Gch1	Kctd9
Adora3	Cldnd1	Gch1	Kif3b
Adprhl1	Clecsf9	Gcnt2	Kif3c
Adrm1	Cln5	Gnal1	Kif3c
Aff1	Cnot4	Gna-rs1	Klc1
Agfg1	Col4a3bp	Gnb4	Larp1
AI849286	Col4a3bp	Golgb1	Larp-pending
Ak2	Col4a3bp	Gorasp2	Lcp2
Ak3l1	Cpeb4	Gosr2	Leng9
Akirin1	Cpeb4	Gp38	Lilrb4
Alas1	Cpne2	Gp49a	Lmo4
Amz2	Csnk1g1	Gpatch3	LOC100044395
Ap2b1	D12Ertd553e	Gpd2	LOC100044475
Apobec3	D3Ucla1	Gpd2	LOC100046483
Appbp2	D7Bwg0611e	Gpr18	LOC100046741
Arfgef2	Daam1	Gpr85	LOC100046781
Arih1	Daam1	Gsk3b	LOC100046781
Arnt	Dcp1a	Gtf2a1	LOC100046817
Arnt	Dcp1a	Gyk	LOC100047323

Cluster 23 Continued

LOC100048583	Ppfia3	Sh2b2	Tnip1
LOC100048726	Ppp1r12a	Sh3glb1	Tnip1
LOC236864	Ppp1r15b	Slamf1	Traf1
LOC237459	Ppp1r15b	Slamf1	Trim56
LOC381891	Ppp2r1b	Slamf7	Triobp
LOC382157	Prkx	Slc11a2	Triobp
LOC632684	Prkx	Slc15a3	Trip12
LOC635086	Psd4	Slc2a1	Trip12
LOC641240	Psmd10	Slc30a7	Trip12
Lrp10	Psmd10	Slc31a2	Upf1
Lrrc25	Ptafr	Slc4a11	Usp42
Lrrc8c	Ptges	Slc6a6	Usp6nl
Luzp1	Ptgir	Slc7a11	Vapa
Mapk1ip1l	Pum2	Slc7a11	Vav1
Mcoln2	Rab10	Slc7a11	Vim
Med21	Rap1b	Slc7a2	Wapal
Mefv	Rap2c	Slco4a1	Wdr37
Metap1	Rapgef5	Slmo2	Ywhaz
Mettl6	Rapgef5	Smurf1	Zbtb7a
Mfap3l	Rapgef5	Snx10	Zeb1
Mfhas1	Rars	Socs1	Zfp238
Mgat4a	Rasgrp1	Sod2	Zfp281
Micall2	Rassf4	Sp1	Zfp281
Micall2	Rbms1	Src	Zfp513
Mitf	Rbpj	Stat3	Zfp654
Mmp13	Rbpms	Stat3	Zfp654
Mmp14	Rgl1	Stat3	Zfp800
Msn	Rgl1	Stau2	Zfpn1a1
Myd88	Rhbdf2	Stx11	Zmynd15
Myd88	Rhog	Stxbp1	
Nab1	Rhou	Tank	
Napg	Riok3	Tank	
Nat5	Riok3	Tank	
Nfe2l1	Riok3	Tapt1	
Nod2	Rlf	Tax1bp1	
Nploc4	Rnf139	Tbc1d1	
Nrp2	Rnf14	Tbk1	
Nrp2	Rufy3	Thrap2	
Nupr1	Saa3	Ticam1	
Pafah1b1	Sbno2	Timp1	
Pik3r5	Sdccag3	Tlk2	
Pkn2	Senp5	Tlk2	
Plaa	Setd8	Tmem132a	
Pofut1	Setd8	Tmem178	
Poldip3	Setd8	Tmem63b	
Ppap2b	Sfmbt1	Tnfrsf11a	
Ppfia3	Sfpi1	Tnfrsf1b	

Table 3.3. Complete list of transcripts from the 24 clusters shown in Figure 3.3.

Chapter 4. Type I IFN regulates the macrophage response to *Mtb*

4.1. Background

The type I IFNs, which include IFNβ and several subtypes of IFNα, are a family of cytokines that signal through a single shared receptor, the IFNαβR (Stark *et al*, 1998). A key property of type I IFN is its antiviral activity. This is due to the induction of several potent antiviral genes by type I IFN, including the GTPase *Mx1* (myxovirus resistance 1); members of the 2′,5′-oligoadenylate synthetase (OAS) family, *Oas1*, *Oas2*, *Oas3* and *Oasl*; protein kinase R (encoded by *Prkr*) and the ubiquitin-like protein *Isg15* (IFN-stimulated protein of 15kDa). As a result, mice unable to signal through type I IFN (*Ifnar1*$^{-/-}$ mice) are profoundly susceptible to certain viral infections (Muller *et al*, 1994).

More recently, a number of studies have shown that type I IFN plays a role in bacterial infection (Decker *et al*, 2005; Trinchieri, 2010). However, the role of type I IFN in bacterial infection is complex, and not always beneficial to the host. *In vitro*, type I IFN can limit the replication of certain bacteria, including *Chlamydia psittaci* and *Chlamydophila pneumonia* (Carlin *et al*, 1989; Buss *et al*, 2010) and *Legionella pneumophila* (Schiavoni *et al*, 2004). Type I IFN can also induce killing of the parasitic protozoan *Leishmania major*, both in macrophages and *in vivo*, through the induction of iNOS (Diefenbach *et al*, 1998; Mattner *et al*, 2000). In addition, *Ifnar1*$^{-/-}$ mice have been shown to be more susceptible to infection with group B streptococci, *Escherichia. coli* and *Streptococcus pneumoniae*, showing that type I IFN enhances the immune response to these bacterial pathogens (Mancuso *et al*, 2007).

In other bacterial infections, however, type I IFN production leads to a reduction in host resistance (Decker *et al*, 2005; Trinchieri, 2010). This was first shown for *Listeria monocytogenes* infection, with *Ifnar1*$^{-/-}$ mice having reduced bacterial burdens in the spleen and liver following infection, compared to WT

controls (Auerbuch *et al*, 2004; Carrero *et al*, 2004; O'Connell *et al*, 2004). Type I IFN in this context was reported to promote the apoptosis of lymphocytes in the spleen, which subsequently led to the induction of IL-10 and a suppression of the immune response to *Listeria monocytogenes* (Carrero *et al*, 2004; O'Connell *et al*, 2004; Carrero *et al*, 2006). More recently, a novel mechanism was proposed whereby type I IFN inhibited the responsiveness of *Listeria monocytogenes*-infected macrophages to IFNγ (Rayamajhi *et al*, 2010).

A number of studies have shown that type I IFN plays a negative role in *Mtb* infection. *Ifnar1*$^{-/-}$ mice are better protected against *Mtb* infection, with reduced bacterial loads in the lung or spleen following infection (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007). In addition, treatment of *Mtb*-infected mice with recombinant IFNαβ led to dramatically reduced survival (Manca *et al*, 2001). However, how type I IFN inhibits the immune response to *Mtb* is poorly understood. More recently, Antonelli *et al* (2010) used Poly-ICLC, a molecule that induces large amounts of type I IFN via TLR3, to study the role of type I IFN in *Mtb*. Treatment of *Mtb*-infected mice with Poly-ICLC led to increased bacterial loads in the lung, and reduced survival, in a type I IFN-dependent manner (Antonelli *et al*, 2010). This was associated with the CCR2-dependent influx of an *Mtb*-permissive population of myeloid cells into the lung (Antonelli *et al*, 2010). Type I IFN has also been reported to suppress the production of the vital pro-inflammatory cytokines IL-1α and IL-1β from macrophages and DCs in the lungs of *Mtb*-infected mice (Mayer-Barber *et al*, 2011). This shows that type I IFN can affect the immune response to bacterial pathogens in a variety of ways.

A recent study has suggested that type I IFN may play a role in human TB infection. Berry *et al* (2010) found that patients with active TB had a signature of

393 genes in the blood, which distinguished them from individuals with latent TB or healthy controls. This signature was significantly associated with both type I IFN and IFNγ signalling, and was found predominantly in neutrophils and monocytes in the blood (Berry *et al*, 2010). However, what effect type I IFN has on the outcome of TB in human patients is unclear.

We were prompted to investigate how type I IFN affected the macrophage response to *Mtb* following the observation that many type I IFN inducible genes were upregulated in macrophages following infection (see previous chapter). We found that endogenous type I IFN regulated the production of many genes from *Mtb*-infected macrophages, including *Nos2*, *Ccl2* and *Ccl7*. Furthermore, addition of type I IFN was shown to regulate the production of cytokines from *Mtb*-infected macrophages, promoting the production of the suppressive cytokine IL-10, but inhibiting the production of the protective cytokines IL-1β, TNFα and IL-12p40. These effects may go some way to explain the detrimental role of type I IFN during *Mtb* infection.

4.2. Results

4.2.1. Endogenous type I IFN promotes IL-10, IL-12p40 and TNFα from *Mtb*-infected macrophages, but inhibits IL-1β

As results in the previous chapter, showing induction of IFNβ mRNA and type I IFN inducible genes, strongly suggested that type I IFN was produced by macrophages in response to *Mtb*, we began by investigating the effects of endogenous type I IFN on macrophage function by deriving macrophages from the bone marrow of *Ifnar1*$^{-/-}$ mice, which are unable to respond to type I IFN. Type I IFN has been shown to affect the production of cytokines, including IL-10, IL-12 and IL-1, from macrophages treated with TLR ligands (Gautier *et al*, 2005; Chang *et al*, 2007; Guarda *et al*, 2011). To determine if autocrine type I IFN also regulated macrophage cytokine production in response to *Mtb*, macrophages derived from WT and *Ifnar1*$^{-/-}$ mice were infected with *Mtb* and cytokine production was determined by ELISA at 24hr post-infection.

In response to *Mtb* infection, WT macrophages produced low levels of IL-12p40, and significant levels IL-10, TNFα and IL-1β, as previously reported (Giacomini *et al*, 2001) but did not produce IL-12p70 (Figure 4.1). However, production of IL-12p40 from *Mtb* infected macrophages was significantly lower in *Ifnar1*$^{-/-}$ macrophages, showing that endogenous type I IFN is required for the production of low levels of IL-12p40 (Figure 4.1). The same trend was observed for IL-10, which was around 4-fold lower in *Ifnar1*$^{-/-}$ macrophages (Figure 4.1). In contrast, *Mtb* induced much higher levels of IL-1β in *Ifnar1*$^{-/-}$ macrophages, showing that endogenous type I IFN suppresses production of IL-1β (Figure 4.1), as previously reported (Novikov *et al*, 2010; Guarda *et al*, 2011; Mayer-Barber *et al*, 2011). However, levels of the important pro-inflammatory cytokine TNFα were

around 3-fold lower in the absence of type I IFN signalling (Figure 4.1). This shows that endogenous type I IFN can regulate the production of both pro- and anti-inflammatory cytokines from *Mtb*-infected macrophages.

4.2.2. Endogenous type I IFN regulates transcription in *Mtb* infected macrophages

Having shown that endogenous type I IFN could affect cytokine production from macrophages, we next investigated whether the global transcriptional response of macrophages to *Mtb* was affected by endogenous type I IFN, as type I IFN can regulate the transcription of many hundreds of genes (Der *et al*, 1998). To investigate this in an unbiased fashion, we used microarray analysis to compare the transcriptional response of WT and *Ifnar1*$^{-/-}$ macrophages. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* and RNA was harvested at 0hr and 6hr post-infection. The RNA was then purified, amplified and hybridised to Illumina Mouse WG-6 V2.0 Beadchip arrays, as described in the Materials and Methods, with triplicate biological samples for each condition. 6hr post-infection was chosen for this analysis, as this was the peak of transcription for most genes following *Mtb*-infection, and also the time point when the majority of IFN-inducible genes were upregulated (see Chapter 3). Following background subtraction, GeneSpring was used to further analyse gene expression.

After normalisation and the removal of undetectable transcripts, as described in the Materials and Methods, fold-change and statistical filters were used to generate differentially regulated transcripts. Transcripts were retained if i) they were induced by 2-fold by *Mtb* in either WT or *Ifnar1*$^{-/-}$ macrophages, ii) they were significantly regulated between WT and *Ifnar1*$^{-/-}$ macrophages as determined by two-

way ANOVA ($p<0.05$) with Benjamini Hochberg FDR multiple testing correction and iii) they were at least 2-fold different between WT and *Ifnar1*$^{-/-}$ macrophages infected with *Mtb* at 6hr post-infection. This left 844 transcripts, which were clustered hierarchically using Pearson centred distance metric.

As shown in Figure 4.2, around half of the 844 transcripts were regulated positively by type I IFN in response to *Mtb* infection. The expression of these transcripts was greatly reduced in *Ifnar1*$^{-/-}$ macrophages compared to WT controls (Figure 4.2). A proportion of these were also strongly regulated by type I IFN at 0hr, and in the medium controls at 6hr, suggesting that macrophages produce basal type I IFN to maintain constitutive gene expression (Figure 4.2). Figure 4.2 also shows that endogenous type I IFN can negatively regulate gene expression in response to *Mtb*, as a number of transcripts were upregulated to a greater extent in *Ifnar1*$^{-/-}$ macrophages compared to WT. In addition, two further clusters showed that type I IFN can affect the downregulation of genes. One cluster was downregulated only in WT macrophages, whereas another was downregulated only in *Ifnar1*$^{-/-}$ macrophages (Figure 4.2). This shows that type I IFN can have both positive and negative effects on *Mtb*-induced transcription in macrophages.

To analyse these different clusters in more detail, *k*-means clustering was used to separate the 844 transcripts into six clusters based on their expression profile, using Pearson centred distance metric. Six clusters were chosen based on the fact that six major groups of transcripts could be distinguished by the dendrogram in Figure 4.2. Heat-maps and expression plots of these six clusters are shown in Figure 4.3, along with a selection of relevant genes from each cluster. Complete lists of the transcripts found in each cluster are presented in section 4.5 at the end of this Chapter.

Figure 4.3 shows that three of the six clusters consisted of transcripts that were positively regulated by type I IFN following *Mtb* infection (clusters 0-2). However, these clusters varied in their dependence upon type I IFN. Strikingly, transcripts in cluster 1 were strongly regulated by type I IFN at 0hr and in uninfected macrophages at 6hr, showing that expression of these transcripts is induced by low levels of type I IFN at baseline (Figure 4.3). *Mtb* infection led to a further upregulation of the transcripts in cluster 1 at 6hr post-infection, and this was dependent upon endogenous type I IFN (Figure 4.3). This shows that *Mtb* induces additional type I IFN production above that produced at baseline, and that this stimulates further gene induction. The genes in Cluster 1 included many antiviral genes, such as *Mx1*, *Mx2, Oas1a, Oas1b, Oas1g, Prkr* and *Samhd1* (Sadler and Williams, 2005), suggesting that type I IFN may promote a constitutive antiviral state in macrophages. Cluster 1 also contained type I IFN-dependent members of the TRIM protein family, including *Pml*, *Trim14*, *Trim21* and *Trim30* (Rajsbaum *et al*, 2008; McNab *et al*, 2010) (Figure 4.3). In addition, several components of the MHC class I antigen presentation pathway were found in cluster 1, suggesting that type I IFN may promote antigen presentation. This included *Tap1*, a component of the TAP transporter which transports cytosolic antigens into the ER, and two proteasome subunits *Psmb7* and *Psmb8*. The transcription factors *Stat1* and *Stat2*, and the antimicrobial effector *Irgm1* (which encodes LRG-47) were also found in cluster 1 (Figure 4.3).

In contrast to cluster 1, transcripts in clusters 0 and 2 were relatively unaffected by basal type I IFN, showing similar expression in WT and *Ifnar1*$^{-/-}$ macrophages at 0hr (Figure 4.3). However, upregulation of these transcripts by *Mtb* at 6hr post-infection was abrogated in *Ifnar1*$^{-/-}$ macrophages (Figure 4.3). Genes in

cluster 0 included *Nos2* (encoding for the enzyme iNOS), the cytokine *Il18* and the chemokines *Cxcl9* and *Cxcl10*. Cluster 0 also contained the TRIM family member *Trim26*, showing that different members of this family can be differentially regulated by basal type I IFN. Genes in cluster 2 included the cytokine *Il27* and the chemokines *Ccl2*, *Ccl5* and *Ccl7* (Figure 4.3). In addition, both cluster 0 and cluster 2 contained genes related to apoptosis. These included *Apaf1* (apoptotic peptidase activating factor 1) and *Casp7* (caspase 7) (Figure 4.3). This may suggest that type I IFN regulates the induction of apoptosis, as previously reported in the context of *Listeria monocytogenes* infection (Carrero *et al*, 2004; Carrero *et al*, 2006). Taken together, these results show that endogenous type I IFN positively regulates a wide range of genes in *Mtb*-infected macrophages, and this includes genes involved in a number of biological processes including the antiviral response, antigen presentation and apoptosis.

The remaining three clusters of transcripts (cluster 3-5) showed very different expression profiles to the genes in clusters 0-2. Transcripts in cluster 4 were upregulated to much higher levels in *Ifnar1*$^{-/-}$ macrophages compared to WT, showing that type I IFN suppresses the upregulation of these transcripts (Figure 4.3). In contrast, transcripts in cluster 3 were downregulated to greater extent in *Ifnar1*$^{-/-}$ macrophages compared to WT. Type I IFN therefore acts to prevent the downregulation of these transcripts (Figure 4.3). Finally, transcripts in cluster 5 were downregulated in a type I IFN-dependent manner, as no downregulation was observed in *Ifnar1*$^{-/-}$ macrophages (Figure 4.3). Several of the genes in these clusters have links to cell-division, including *Ccng2* (Cyclin G2), which is part of cluster 3; *Cdkn2b* (cyclin-dependent kinase inhibitor 2B), part of cluster 4 and *Skp2* (S-phase

kinase-associated protein 2), in cluster 5 (Figure 4.3). This may suggest that type I IFN regulates cell-division.

To further investigate the functions of these six clusters, each was analysed by GO analysis and IPA (Figure 4.4). As expected, the genes in cluster 0-2 were associated with IFN signalling by IPA (Figure 4.4). Cluster 1 showed significant overlap with the IFN signalling canonical pathway, with cluster 0 also linked to "Role of Jak1, Jak2 and Tyk2 in interferon signalling" and "Jak/Stat signalling" (Figure 4.4). Cluster 0 was also linked to IL-15 signalling, reflecting the presence of *Il15* and *Il15ra* in this cluster (Figure 4.4). However, no significant overlap with GO terms or IPA pathways was observed for the genes in clusters 3-5 (Figure 4.4). Further analysis will be required to determine the functions of these genes.

4.2.3. Exogenous type I IFN promotes IL-10 production from *Mtb*-infected macrophages, but inhibits IL-12p40, TNFα and IL-1β

Results presented in Figure 4.1 showed that type I IFN produced by macrophages in response to *Mtb* infection could feed back and regulate the production of cytokines; positively regulating IL-10, IL-12p40 and TNFα but negatively regulating IL-1β. However, many cells types can produce type I IFN during infection, such as fibroblasts, myeloid DCs and pDCs (Theofilopoulos *et al*, 2005). During *Mtb* infection *in vivo*, type I IFN produced by other cell types could therefore act on infected macrophages. We therefore investigated whether exogenous type I IFN could also regulate cytokine production from *Mtb*-infected macrophages, by infecting macrophages with *Mtb* in the presence or absence of increasing doses of recombinant murine IFNβ. The production of cytokines was determine by ELISA at 24hr post-infection (Figure 4.5A). In addition, RNA was harvested from

macrophages infected with *Mtb* with or without IFNβ and analysed by qPCR, to determine if IFNβ regulated cytokine production at the mRNA level (Figure 4.5B). Based on the results in Figure 4.5A, a dose of 2ng/ml of IFNβ was used for the qPCR experiments in Figure 4.5B.

IL-12p40 protein production from *Mtb*-infected macrophages was significantly suppressed by addition of IFNβ (Figure 4.5A). This was despite the fact that endogenous type I IFN is required for maximum IL-12p40 production (Figure 4.1) and demonstrates that endogenous and exogenous type I IFNs can have opposite effects. Addition of IFNβ did not suppress IL-12p40 at the mRNA level, suggesting that post-transcriptional regulation is likely to be involved (Figure 4.5B). IL-12p40 plays an important role *in vivo* in the immune response to *Mtb*, stimulating DCs to migrate to the draining lymph nodes and activate the protective $CD4^+$ T cell response (Khader *et al*, 2006). Suppression of IL-12p40 production by IFNβ may therefore delay the activation of this response.

Although IL-12p70 production was below the limit of detection at the protein level, IFNβ suppressed *Il12a* mRNA (Figure 4.5B), which encodes the p35 subunit of IL-12p70. This suggests that IFNβ may be able to inhibit IL-12p70 production from cells that produce IL-12p70 in significant amounts, such as DCs, when appropriately stimulated.

In keeping with the effects of endogenous type I IFN, addition of IFNβ enhanced IL-10 protein production from macrophages in a dose-dependent manner (Figure 4.5A). Although small effects were seen with the addition IFNβ at low doses (20pg and 200pg/ml) a significant effect was only observed with doses of 2ng/ml and higher (Figure 4.5A). Importantly, addition of IFNβ at 2ng/ml in the absence of *Mtb* did not induce IL-10 production, demonstrating that IFNβ enhances IL-10

production in concert with another stimulus, rather than inducing IL-10 itself (Figure 4.5A). IFNβ also promoted IL-10 mRNA production (Figure 4.5B). In response to *Mtb* alone, *Il10* mRNA was rapidly induced, peaking at 1hr post-infection, but then rapidly returned to baseline levels at 6hr post-infection (Figure 4.5B). Addition of IFNβ led to sustained IL-10 mRNA transcription, with significantly higher levels seen at 6hr post-infection compared to untreated controls (Figure 4.5B). This suggests that in the context of *Mtb* infection, type I IFN could promote IL-10 production, which may lead to a suppression of the immune response.

Also in agreement with the effect of endogenous type I IFN, IFNβ dramatically inhibited IL-1β protein production from *Mtb* infected macrophages (Figure 4.5A). This is in keeping with previous studies (Novikov *et al*, 2010; Guarda *et al*, 2011; Mayer-Barber *et al*, 2011). A significant reduction was seen with the lowest dose of 20pg/ml of IFNβ, and IL-1β protein production was dramatically suppressed with higher IFNβ doses (Figure 4.5A). IFNβ suppressed transcription of *Il1b* mRNA at 6hr and 24hr post-infection (Figure 4.5B), showing that type I IFN can inhibit the transcription of pro-IL-1β, as reported by Guarda *et al* (2011) in macrophages stimulated with various inflammasome activating stimuli such as LPS and alum. However, this study also showed that type I IFN could suppress the activation of the inflammasome in macrophages, suggesting that IFNβ may have multiple mechanisms to suppress IL-1β production (Guarda *et al*, 2011).

IFNβ also inhibited TNFα production from infected macrophages, with a significant effect seen at doses of 2ng/ml and higher (Figure 4.5A). Suppression of TNFα and IL-1β by type I IFN may have negative consequences *in vivo*, as both are required for protection from *Mtb* (Flynn *et al*, 1995; Mayer-Barber *et al*, 2011).

However, addition of IFNβ did not affect the transcription of *Tnf*, suggesting that post-transcriptional mechanisms may be involved (Figure 4.5B).

As a control, we next determined whether the effects of IFNβ on cytokine production were dependent on the IFNαβR. This would rule out the possibility that our IFNβ preparation was contaminated with PRR ligands, as such effects would occur independently of the IFNαβR. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* with or without IFNβ at 2ng/ml, and cytokine production determined at 24hr post-infection. As shown in Figure 4.6, IFNβ promoted IL-10, but inhibited IL-12p40, TNFα and IL-1β from *Mtb*-infected macrophages, in keeping with previous results. However, these effects were totally dependent on the IFNαβR, as no change in cytokine production in response to IFNβ was observed in *Ifnar1*$^{-/-}$ macrophages (Figure 4.6). This confirms that IFNβ regulates cytokine production through type I IFN receptor signalling, and rules out any possible effects of contamination.

Taken together, these results show that exogenous IFNβ can affect the production of cytokines from *Mtb*-infected macrophages, and that this probably occurs via both transcriptional and post-transcriptional mechanisms. These results would be predicted to have a negative effect on *Mtb* infection *in vivo*, as IFNβ promoted the suppressive cytokine IL-10, whilst inhibiting the protective cytokines IL-12p40, TNFα and IL-1β.

4.2.4. Effects of type I IFN on cytokine production are not due to regulation of *Mtb* survival

One possible explanation for the effects of type I IFN on cytokine production could be that type I IFN affects *Mtb* survival within macrophages, thus changing the amount of PRR stimulation and the subsequent induction of cytokines. To address

this, the effect of both endogenous and exogenous type I IFN on *Mtb* survival in macrophages was determined by serial dilution and plating, as described in the Materials and Methods. 96hr post-infection was chosen for this time point, as this was when an effect of IFNγ on *Mtb* survival was observed.

As shown in Figure 4.7A, addition of IFNβ at 2ng/ml had no effect on the levels of *Mtb* within macrophages at 96hr post-infection. In addition, comparison of bacterial levels in WT compared to *Ifnar1*$^{-/-}$ macrophages at 96hr showed that endogenous type I IFN did not affect *Mtb* survival (Figure 4.7B). This shows that type I IFN modulates cytokine production independently of effects on *Mtb* survival.

4.2.5. IFNβ suppresses pro-inflammatory cytokine production in part via autocrine IL-10

IL-10 is a potent anti-inflammatory cytokine (Moore *et al*, 2001) and has been shown to inhibit IL-12, IL-1β and TNFα production from APCs (Fiorentino *et al*, 1991a; Fiorentino *et al*, 1991b; D'Andrea *et al*, 1993). We found that addition of IFNβ potently enhanced IL-10 production from infected macrophages. It was therefore possible that IFNβ may suppress the production of IL-12, IL-1β and TNFα indirectly, through increasing the amount of IL-10 acting on the cells.

The ability of IFNβ to affect IL-12, IL-1β and TNFα was therefore assessed in the absence of IL-10, using *Il10*$^{-/-}$ macrophages. WT and *Il10*$^{-/-}$ macrophages were infected with *Mtb* in the presence or absence of IFNβ. In WT macrophages, IFNβ again suppressed IL-12p40, IL-1β and TNFα, whilst enhancing IL-10 (Figure 4.8), as previously shown. As expected, in *Il10*$^{-/-}$ macrophages levels of the pro-inflammatory cytokines IL-12p40, TNFα and to a lesser extent IL-1β were increased, showing that endogenous IL-10 is capable of inhibiting production of these

cytokines (Figure 4.8). Importantly, the suppressive effect of exogenous IFNβ on IL-12p40 production was diminished in *Il10*$^{-/-}$ macrophages, showing that increased IL-10 production is the major mechanism behind the suppression. However, a significant decrease in IL-12p40 production in response to IFNβ was still observed in *Il10*$^{-/-}$ macrophages, suggesting additional IL-10-independent mechanisms (Figure 4.8).

In the case of TNFα however, the suppressive effect of IFNβ was completely lost in *Il10*$^{-/-}$ macrophages (Figure 4.8). Whereas in WT macrophages IFNβ suppressed TNFα by around 2-fold, IFNβ had no effect in the absence of IL-10, showing that IFNβ suppresses TNFα entirely through enhancing IL-10 production (Figure 4.8).

In contrast, IFNβ still effectively suppressed IL-1β production in *Il10*$^{-/-}$ macrophages, meaning that this effect is independent of IL-10 (Figure 4.8). In WT and *Il10*$^{-/-}$ macrophages, IFNβ suppressed IL-1β by around 3-fold (Figure 4.8). This is in agreement with the fact that IL-1β seems generally less sensitive to the suppressive effects of autocrine IL-10, as there was a relatively small increase in IL-1β levels between WT and *Il10*$^{-/-}$ macrophages infected with *Mtb* (Figure 4.8). This shows that IFNβ suppresses IL-12p40 and TNFα through increasing IL-10 production, but that the effect of IFNβ on IL-1β is IL-10-independent.

4.2.6. Type I IFN regulates IL-27 production

The previous results show that the effects of IFNβ on IL-12p40 and TNFα occur indirectly, via increased IL-10 production. We therefore investigated further the mechanism by which IFNβ enhances IL-10 production from *Mtb*-infected macrophages. A previous study has shown that IFNβ can upregulate IL-27

production from LPS treated macrophages, and that IL-27 was responsible for increasing IL-10 (Iyer *et al*, 2010). As we had previously observed by microarray that *Il27* mRNA was decreased in *Ifnar1*$^{-/-}$ macrophages compared to WT, we investigated whether endogenous and exogenous type I IFN could promote IL-27 production.

WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* and production of IL-27 was determined at 24hr post-infection. As shown in Figure 4.9A, IL-27 was induced by *Mtb* in WT macrophages. However, this was completely dependent on endogenous type I IFN, with no IL-27 detectable in *Ifnar1*$^{-/-}$ macrophages (Figure 4.9A). To determine if exogenous IFNβ could also promote IL-27 production, WT macrophages were infected with *Mtb* in the presence or absence of IFNβ. Figure 4.9B shows that addition of IFNβ enhanced IL-27 production by around 2-fold from WT macrophages. These results demonstrate that both endogenous and exogenous type I IFN can promote IL-27 production from *Mtb*-infected macrophages. This presents a possible mechanism for the induction of IL-10 by type I IFN. However, further experiments using macrophages deficient for the IL-27 receptor will be required to confirm whether the enhancement of IL-27 accounts for the increases in IL-10 production stimulated by type I IFN.

4.2.7. Pre-treatment of macrophages with IFNβ leads to an upregulation of IL-12p40 and IL-12p70

Whereas endogenous type I IFN enhanced IL-12p40 production (Figure 4.1), exogenous IFNβ suppressed it (Figure 4.5A). One possible explanation was that the dose of type I IFN could have different effects; a low amount of endogenous type I IFN could promote IL-12p40, whereas the high exogenous dose of 2ng/ml could

suppress IL-12p40. However, Figure 4.5 shows that even much lower doses of exogenous IFNβ suppressed IL-12p40, suggesting that dose was not the reason. Another possibility was length of exposure to type I IFN. The results presented in Figure 4.2 strongly suggested that low levels of basal type I IFN are present at baseline. As macrophages are rested overnight prior to infection (see Materials and Methods) they would be exposed to this basal type I IFN for a significant length of time prior to infection. In contrast, exogenous IFNβ was always added at the same time as infection.

To determine if length of exposure altered the effects of IFNβ on the production of IL-12p40 and other cytokines, macrophages were pre-treated with IFNβ for 12hr, 8hr, 4hr and 2hr prior to *Mtb* infection, with IFNβ added at 0hr as a control. *Mtb* only controls were included for each pre-incubation time to control for the effect of resting the macrophages for varying lengths of time prior to infection.

As previously shown, IL-12p40 production was suppressed by addition of IFNβ at 0hr (Figure 4.10). This effect was also seen with addition of IFNβ at 2hr and 4hr prior to infection (Figure 4.10). However, the effect was completely reversed by addition of IFNβ at 8hr and 12hr before infection, with IFNβ stimulating a dramatic increase of IL-12p40 (Figure 4.10). At 12hr this increase was greater than 10-fold compared to *Mtb* alone (Figure 4.10). Pre-treatment with IFNβ for 12hr also induced IL-12p70, although at barely detectable levels (Figure 4.10). Production of IL-12p70 was notable, as it was not detectable from macrophages under other conditions. This shows that IFNβ can have opposite effects on IL-12 production from macrophages, depending on the time IFNβ is added relative to *Mtb*.

In contrast to IL-12p40, IFNβ consistently upregulated IL-10 production, regardless of the time of addition, although this was most effective if IFNβ was

added at 0hr or 2hr (Figure 4.10). Of note, IFNβ upregulated IL-10 when added at 8hr and 12hr, despite simultaneously promoting IL-12 production (Figure 4.10). This suggests that, when added at 8hr and 12hr prior to infection, IFNβ activates additional mechanisms to promote IL-12, and overcome the suppressive effects of IL-10.

The effect of IFNβ on IL-1β was most effective when added at 0hr, and became steadily less effective at longer pre-treatment times (Figure 4.10). IFNβ had no significant effect if added at 12hr prior infection, and only a minimal effect if added 8hr prior to infection (Figure 4.10). This may suggest that the mechanisms for IFNβ suppression of IL-1β are activated transiently. TNFα was also suppressed more effectively by IFNβ if added closer to the time of infection (Figure 4.10). If added at 2hr and 4hr prior to infection, IFNβ significantly suppressed TNFα. However, no significant effect was seen with IFNβ added at 8hr or 12hr prior to infection (Figure 4.10). These results show that pre-treatment of macrophages with IFNβ can dramatically alter the effect on cytokine production in response to *Mtb*.

4.3. Discussion

Type I IFN is known to play a detrimental role in the immune response to *Mtb*, as mice deficient in type I IFN have lower bacterial loads following infection (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007). In addition, treatment of *Mtb*-infected mice with recombinant IFNαβ (Manca *et al*, 2001) or the type I IFN inducing molecule Poly-ICLC (Antonelli *et al*, 2010) led to reduced survival. Here we characterised the regulation of the macrophage response to *Mtb* by type I IFN. We found that both cytokine production and transcription were potently regulated by type I IFN. The effects of endogenous and exogenous type I IFN on cytokine production by *Mtb*-infected macrophage are summarised in Figures 4.11 and 4.12.

4.3.1. The regulation of the macrophage response by endogenous type I IFN

Following the observation of a type I IFN signature in *Mtb*-infected macrophages, it was important to confirm that these genes were type I IFN dependent, and also to determine the global effects of type I IFN on gene expression in response to *Mtb*. Using microarray analysis of WT vs. *Ifnar1*$^{-/-}$ macrophages infected with *Mtb*, we found 844 transcripts that were regulated by endogenous type I IFN. These genes were regulated in a variety of ways. Some genes were dependent upon type I IFN for their expression, whilst other were induced to a greater extent in *Ifnar1*$^{-/-}$ macrophages. This shows that type I IFN both induces and represses the transcription of genes.

It was striking that the basal expression of many genes was lost in *Ifnar1*$^{-/-}$ macrophages, suggesting that low level constitutive type I IFN was produced by macrophages. The importance of constitutive type I IFN production has been

appreciated for many years (reviewed in Gough *et al*, 2012). However, our results show that different type I IFN inducible genes showed varying susceptibility to basal type I IFN, with many genes not affected by the loss of type I IFN at baseline. In general, antiviral genes such as *Mx1*, *Oas* and *Prkr* were strongly regulated by basal type I IFN, whereas more immune related genes such as *Nos2*, *Cxcl10* and *Ccl2* were not. This makes sense; whereas constitutive expression of antiviral genes may lead to a more rapid antiviral response, constitutive expression of pro-inflammatory genes such as *Nos2*, *Cxcl10* and *Ccl2* would be predicted to cause inappropriate inflammation. However, the mechanism behind this differing responsiveness is unclear. It may be that different genes respond to differing levels of type I IFN. Basally regulated genes may require relatively little type I IFN for their induction, whereas genes such as *Nos2* require the increased levels generated following *Mtb* infection. Alternatively, induction of genes such as *Nos2* may require addition transcriptional regulators besides those induced by type I IFN. For example, it was recently shown that both ISGF3 and NF-κB are required for *Nos2* transcription in *Listeria* infected macrophages (Farlik *et al*, 2010). Thus type I IFN alone would be insufficient for *Nos2* induction, with *Nos2* only being expressed following the activation of NF-κB in response to *Mtb* infection.

Many immunologically important genes were regulated by endogenous type I IFN in macrophages. These included *Nos2*, as discussed above, as well as *Irgm1*, which encodes for LRG-47 and the chemokines *Ccl2*, *Ccl7* and *Cxcl10* (IP-10). These genes are all important for protection against *Mtb*. Both iNOS and LRG-47 are crucial for the killing of *Mtb* in macrophages in response to IFNγ, and mice deficient in either gene cannot control *Mtb* infection (Chan *et al*, 1995; MacMicking *et al*, 2003). *Ccl2* and *Ccl7* signal through the chemokine receptor CCR2 on monocytes,

and stimulate their migration from the bone marrow into the site of infection (Shi and Pamer, 2011). *Ccr2* deficient mice do not recruit monocytes to the infected lung, and are unable to control *Mtb* infection (Peters *et al*, 2001). IP-10, in contrast, is required for T cell migration into the lung during infection (Khan *et al*, 2000). The promotion of these genes by type I IFN would therefore be predicted to have a beneficial effect on the host immune response in the context of *Mtb* infection. Indeed, induction of iNOS by type I IFN has been shown to be protective in the context of *Leishmania major* infection (Diefenbach *et al*, 1998; Mattner *et al*, 2000). However, type I IFN has consistently been shown to play a negative role during *Mtb* infection (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007) as discussed above. It is possible that type I IFN has other functions that over-ride these potentially beneficial effects. However, the promotion of genes such as *Ccl2* may not always be beneficial. Antonelli *et al* (2010) showed that induction of type I IFN by Poly-ICLC in *Mtb*-infected mice led to increased *Ccl2* expression, in keeping with our results, but this led to the influx of a permissive myeloid population into the lung, leading to decreased survival. Thus, type I IFN may be a double-edged sword during *Mtb* infection.

It should also be noted that, despite promoting the production of iNOS and LRG-47, type I IFN did not reduce the survival of *Mtb* within macrophages. This was the case both for endogenous and exogenous type I IFN. It is possible that type I IFN does not induce iNOS production to sufficient levels to mediate *Mtb* killing. A more potent activator of macrophages such as IFNγ, which activates additional killing mechanisms such as autophagy, may therefore be required to lead to macrophage killing of *Mtb*. However, we addressed the effect of IFNβ on *Mtb* survival only with adding IFNβ at the time of infection. As shown in Figure 4.10,

type I IFN can be a more potent stimulus when added prior to infection. Future experiments could therefore investigate whether pre-incubation of macrophages with IFNβ could mediate *Mtb* killing.

4.3.2. The regulation of IL-10 by type I IFN

We found that both endogenous and exogenous type I IFN could induce the production of IL-10 from macrophages. This is in keeping with several previous studies. Chang *et al* (2007) found that the induction of IL-10 by LPS in murine macrophages was dependent upon endogenous type I IFN. This study also showed that addition of IFNβ to macrophages could induce IL-10 (Chang *et al*, 2007). However, in this study, addition of IFNβ alone, without a secondary TLR stimulus, was sufficient to induce IL-10, whereas in our system a dose of 2ng/ml IFNβ alone did not induce IL-10; it only enhanced the production of IL-10 in response to *Mtb*. The reasons for this discrepancy are unclear, but may be due to differences in the protocols used to generate and stimulate macrophages. However, given that induction of IL-10 requires several different signalling pathways that are not known to be induced by type I IFN, such as NF-κB and ERK MAP kinase, it would seem likely that in the majority of cases, type I IFN would require a secondary TLR stimulation in order to affect the production of IL-10.

Type I IFNs have also been shown to induce IL-10 in several other cell types. This includes cells of the innate immune system such as human monocytes (Aman *et al*, 1996) and human DCs (Wang *et al*, 2011). In addition, type I IFN has been shown to induce IL-10 from T cells. Levings *et al* (2001) showed that IFNα caused naive $CD4^+$ T cells to differentiate into $IL\text{-}10^+$ T-regulatory cells. More recently, Zhang *et al* (2011) showed that both IFNα and IFNβ could enhance IL-10 production

from Th17 cells, which was associated with a decrease in IL-17 production. The link between type I IFN and IL-10 therefore appears to occur in a number of different cell types, including both innate and adaptive cells, suggesting that this is an important regulatory mechanism.

Various suggestions have been put forward as to the mechanism of IL-10 induction by type I IFNs. Type I IFN induction of IRF1 and STAT3 has been reported to promote IL-10 from a human B-cell line (Ziegler-Heitbrock *et al*, 2003). In addition, type I IFN has been reported to induce IL-10 through glycogen synthase kinase 3 (GSK3) in human DCs. In mouse macrophages, type I IFN has been shown to induce IL-10 via autocrine induction of IL-27 (Iyer *et al*, 2010). We also found that both endogenous and exogenous type I IFN promoted IL-27 production from *Mtb* infected macrophages. *Il27* was also found to be lower at the mRNA level in *Ifnar1*$^{-/-}$ macrophages compared to WT, suggesting transcriptional regulation. We plan to further investigate whether IFNβ upregulates IL-10 production via IL-27 by adding *Mtb* and IFNβ to WT macrophages and macrophages from mice deficient in the receptor for IL-27 (WSX1$^{-/-}$), and determining if IFNβ is still able to upregulate IL-10 production.

More recently, a novel pathway has been implicated in IL-10 production, which may link to type I IFN. Protein kinase R (PKR), a strongly type I IFN inducible antiviral molecule, was shown to mediate IL-10 production in macrophages stimulated with LPS and dsRNA (Chakrabarti *et al*, 2008). Macrophages deficient in PKR were subsequently shown to produce lower levels of IL-10 in response to *Mtb* infection (Wu *et al*, 2012) although this study did not address the role of type I IFN in IL-10 production. In our microarray study, *Prkr* (encoding for protein kinase R) was dramatically lower in *Ifnar1*$^{-/-}$ macrophages,

suggesting that PKR may play a role in IL-10 production. This could be further investigated by adding type I IFN to macrophages from mice deficient in PKR.

IL-10 is known to play a negative role in the immune response to *Mtb in vivo*, resulting in a delayed and reduced Th1 response (Redford *et al*, 2010) and type I IFN has also been reported to inhibit the protective immune response during *Mtb* infection (Manca *et al*, 2005; Ordway *et al*, 2007; Antonelli *et al*, 2010). Our study suggests that a possible mechanism for the negative effects of type I IFN could be in part via induction of IL-10.

4.3.3. Type I IFN regulation of IL-12

IL-12 is an important cytokine in the immune response to *Mtb*, due to its ability to stimulate Th1 development and IFNγ production (Flynn and Chan, 2001a). In *Mtb*-infected macrophages, endogenous type I IFN was required for maximal IL-12p40 production. However, this was in contrast to the fact that addition of exogenous IFNβ could potently suppress IL-12p40 production.

A number of studies have shown that exogenous IFNα or IFNβ can inhibit the production of IL-12 from a variety of cell types, and in response to a variety of stimuli (Cousens *et al*, 1997; McRae *et al*, 1998; Byrnes *et al*, 2001; Dalod *et al*, 2002; Heystek *et al*, 2003). In addition, Cousens *et al* (2007) showed that type I IFN also inhibited IL-12 *in vivo* during infection of mice with murine cytomegalovirus (MCMV), and that antibody blockade of IFNαβ in these mice enhanced early IFNγ production. Two of these studies showed that effects of type I IFN in suppressing IL-12 were IL-10-independent (McRae *et al*, 1998; Byrnes *et al*, 2001) in contrast to our results showing a partial IL-10-dependence. However, these studies were carried out

in human monocytes and DCs, and used single TLR ligands, and it is possible that different mechanisms exist depending on the cell type and stimulus used.

In contrast to the effects of exogenous type I IFN, Gautier *et al* (2005) showed that endogenous type I IFN was required for maximal IL-12p40 and IL-12p70 production from murine DCs, in agreement with our results. DCs from mice lacking the IFNαβR produced markedly less IL-12p70 and IL-12p40 in response to a variety of single TLR ligands. The reason for the discrepancy between endogenous and exogenous type I IFN has not been explained. However, in our study the suppressive effects of IFNβ on IL-12 were reversed if macrophages were pre-treated for 8hr or 12hr with IFNβ. In this case, IFNβ could dramatically upregulate IL-12p40 production. This result could explain the apparent contradictions between endogenous and exogenous type I IFN. It may also have implications for the role of type I IFN *in vivo*; the timing of type I IFN production in response to a pathogen may have opposite effects on IL-12 production, and thus skew the immune response in different directions.

The mechanisms behind these opposing effects of type I IFN are at present unclear. However, IFNβ still increased IL-10 production from infected macrophages if added at 8hr or 12hr prior to infection, suggesting that pre-treatment activates additional, IL-12 stimulatory pathways that can overcome the suppressive effects of IL-10. A good candidate for this effect could be the transcription factor IRF1. IFNγ signals through IRF1 to induce IL-12 production (Liu *et al*, 2004) and type I IFN is also known to induce IRF1 activation (Tamura *et al*, 2008). Studies using IRF1 deficient macrophages could address whether this transcription factor is required for the upregulation of IL-12 by macrophages pre-treated with IFNβ.

As macrophages produce only IL-12p40, and not IL-12p70, in response to *Mtb*, we were unable to assess the effects of IFNβ on IL-12p70 production. However, *Il12a* mRNA was suppressed by IFNβ, suggesting IFNβ could suppress IL-12p70. Future studies could address the effects of IFNβ on IL-12p70 in cells such as myeloid DCs, which are the major producers of IL-12p70 *in vivo* (Rothfuchs *et al*, 2009). Suppression of IL-12p70 by IFNβ could have important consequences for *Mtb* infection, as IL-12p70 is absolutely required for protection, due to its effects in generating the Th1 response (Cooper *et al*, 2011).

4.3.4. Type I IFN suppresses IL-1β production

IL-1α and IL-1β are both critical in protecting the host from *Mtb* infection (Mayer-Barber *et al*, 2011). This requirement for IL-1 signalling explains why MyD88 deficient mice are highly susceptible to *Mtb* infection, as IL-1 signals through MyD88 (Cooper *et al*, 2011). We show that both endogenous and exogenous type I IFN could potently inhibit IL-1β production from *Mtb* infected macrophages. IL-1β was highly sensitive to IFNβ mediated suppression, with amounts as low as 20pg/ml sufficient to inhibit IL-1β production. Suppression of IL-1β may represent a mechanism by which type I IFN increases the susceptibility of mice to *Mtb* infection.

These results are in agreement with a recent study by Guarda *et al* (2011) which reported that both endogenous and exogenous type I IFN could inhibit IL-1β production from macrophages in response to several inflammasome activating stimuli such as LPS and alum. IFNβ suppressed production of pro-IL-1β at the mRNA level in response to LPS, in keeping with our results using *Mtb* (Guarda *et al*, 2011). It was also shown that IFNβ could inhibit the activation of caspase-1 by the inflammasome, although this was specific to the NLRP3 and NLRP1b

inflammasomes, and not the AIM2 or IPAF inflammasomes (Guarda *et al*, 2011). As *Mtb* activates IL-1 production through the NLRP3 inflammasome (Cooper *et al*, 2011) further work should address whether the assembly or activity of the NLRP3 inflammasome is affected by type I IFN in *Mtb*-infected macrophages.

A recent study has also shown that IFNβ inhibits both IL-1α and IL-1β production *in vivo* in mice infected with *Mtb* (Mayer-Barber *et al*, 2011). Type I IFN production suppressed IL-1α and IL-1β from both DCs and macrophages in the lung following *Mtb* infection, and this occurred through direct effects of type I IFN, as mixed bone-marrow chimeras showed that loss of *Ifnar1* on the surface of myeloid cells resulted in increased IL-1 production from these cells (Mayer-Barber *et al*, 2011). This study supports our findings that type I IFN suppresses IL-1β production, and shows that this may have important consequences *in vivo*. Furthermore, it has also been shown that type I IFN suppresses IL-1β production from human macrophages infected with *Mtb* (Novikov *et al*, 2010). Given that active TB patients have an IFN signature in the blood (Berry *et al*, 2010), it is possible that suppression of IL-1β by type I IFN may be occurring in humans with active TB. Further investigation is required to determine what effect this may have on the outcome of infection in TB patients.

4.4. Figures

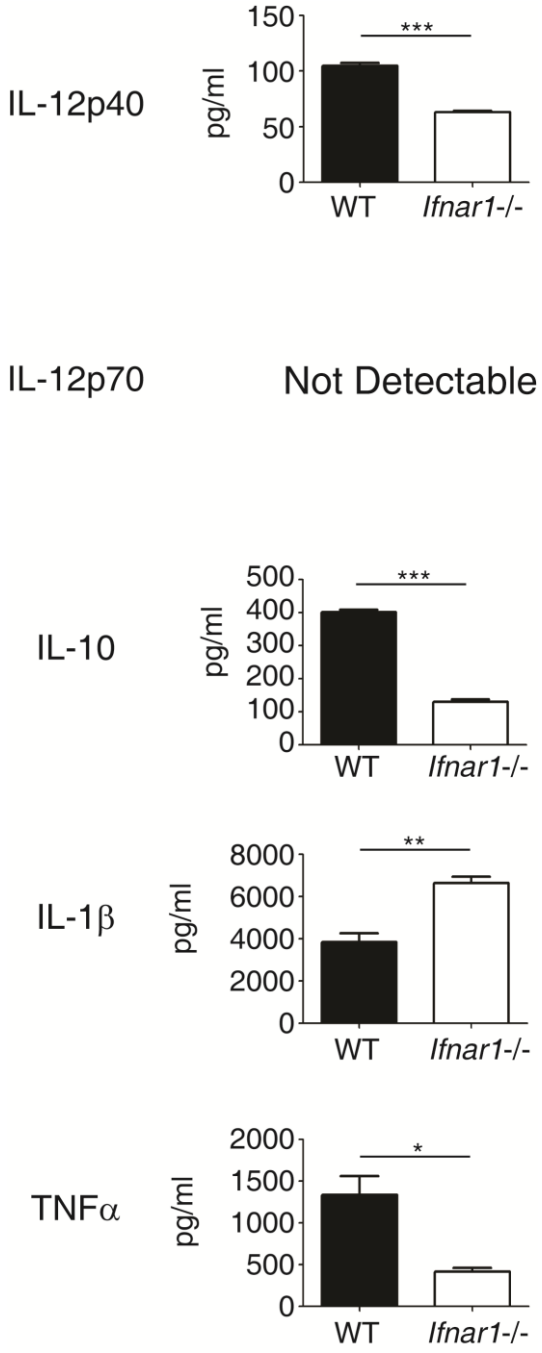

Figure 4.1. Endogenous type I IFN promotes IL-10, IL-12p40 and TNFα, but negatively regulates IL-1β, from *Mtb*-infected macrophages. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb*. Cytokine levels in culture supernatant were determined at 24hr post-infection. Graphs show mean +/- standard error from the mean (SEM) (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; unpaired t-test. Data is representative of at least three independent experiments.

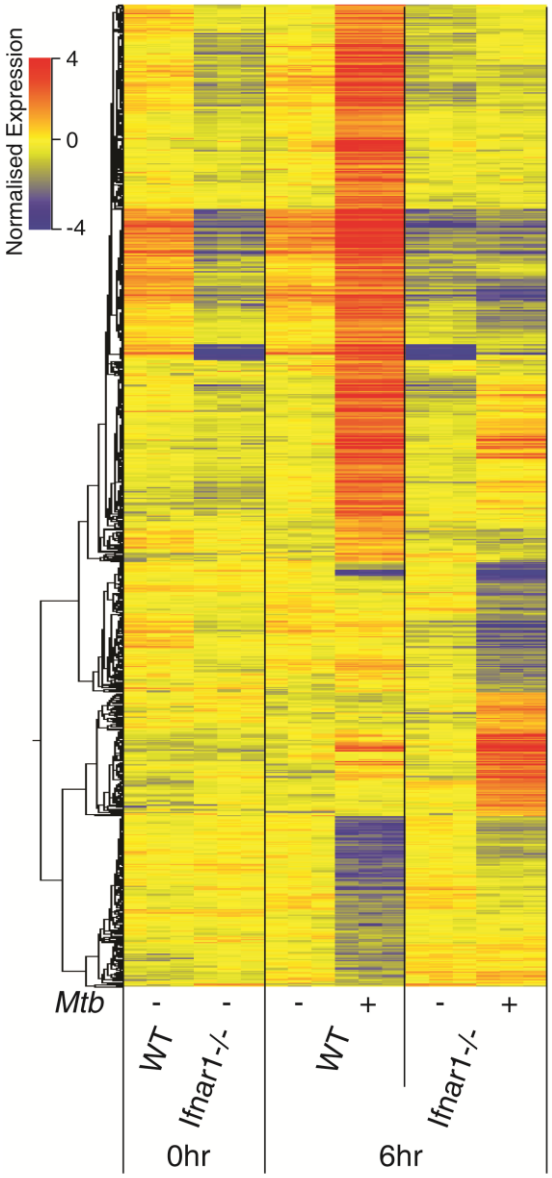

Figure 4.2. Endogenous type I IFN regulates the macrophage transcriptional response to *Mtb*. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb*, RNA was harvested at 0hr and 6hr post-infection and microarray analysis was carried out. Data was normalised and undetectable transcripts were removed as described in the Materials and Methods. Differentially regulated transcripts were obtained by taking those that were at least 2-fold up- or downregulated in infected samples vs. controls in either strain at 6hr post-infection, and those that were significantly different between WT and *Ifnar1*$^{-/-}$ by two-way ANOVA $p<0.05$ with Benjamini Hochberg FDR multiple testing correction. A further filter removed transcripts less than 2-fold different between WT and *Ifnar1*$^{-/-}$ infected samples at 6hr post-infection. This left 844 transcripts, which were subjected to hierarchical clustering with Pearson's centred distance metric and average linkage, and visualised with a heat map.

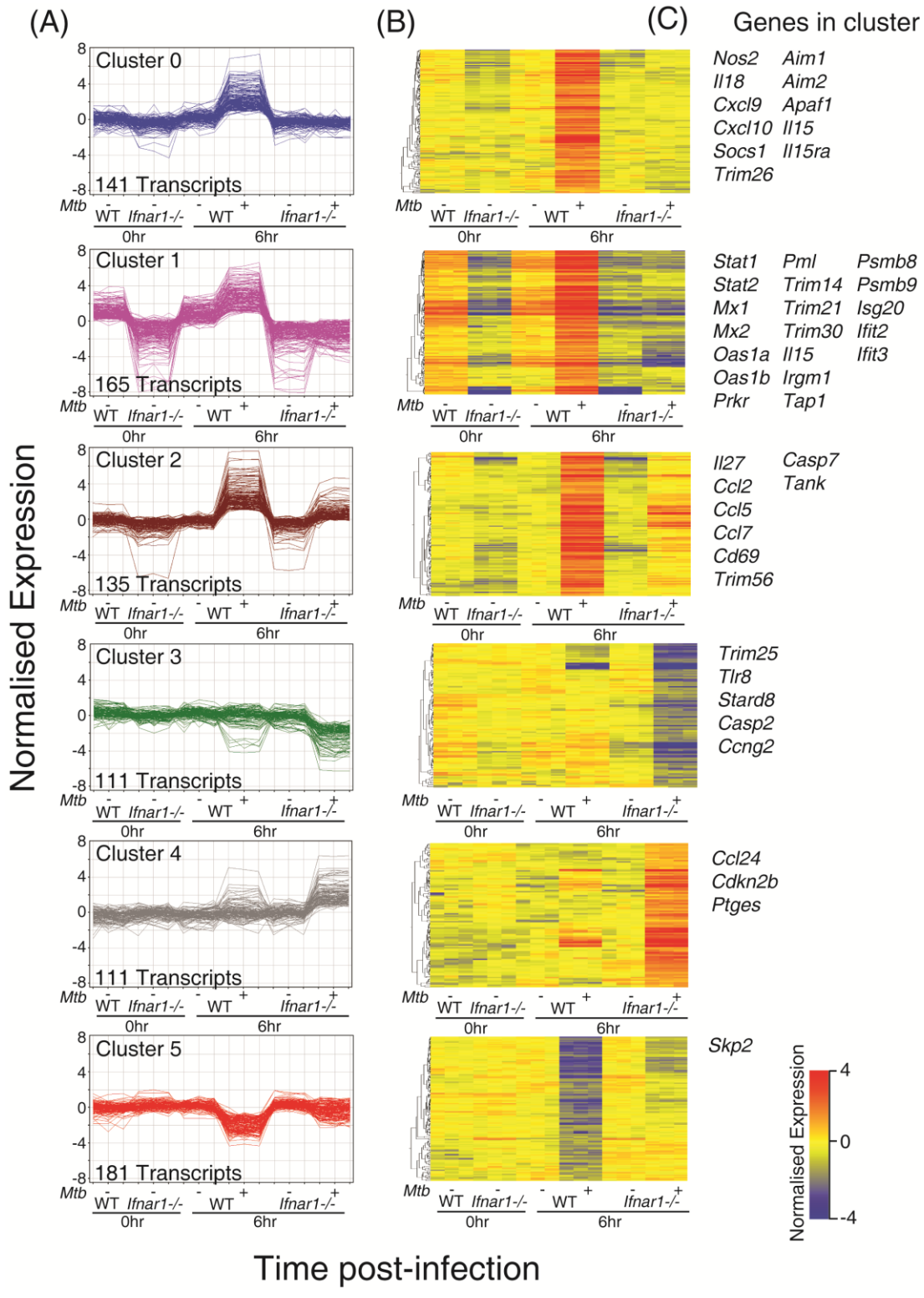

Figure 4.3. Transcripts regulated by endogenous type I IFN in *Mtb*-infected macrophages can be separated into six clusters. The 844 differentially regulated transcripts shown in Figure 4.2 were separated into 6 clusters by *k*-means clustering, with Pearson centred distance metric. (A) Expression profiles and (B) heat maps of the 6 clusters. (C) Selected genes in the 6 clusters.

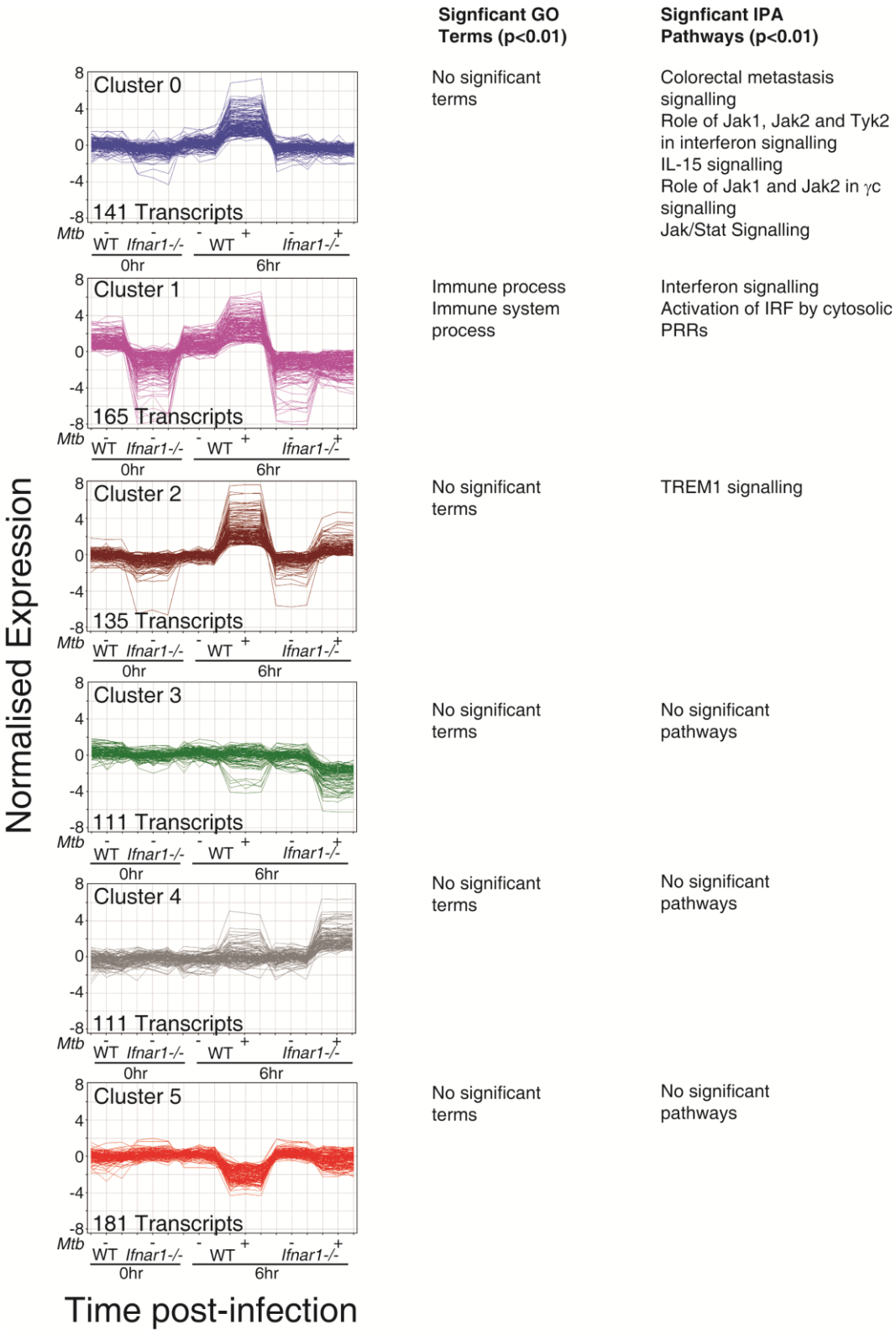

Figure 4.4. Overlap of the six clusters with IPA and GO terms. The transcripts in each of the 6 *k*-means clusters shown in Figure 4.3 were analysed by IPA and GO analysis. Overlap was considered significant if p<0.01.

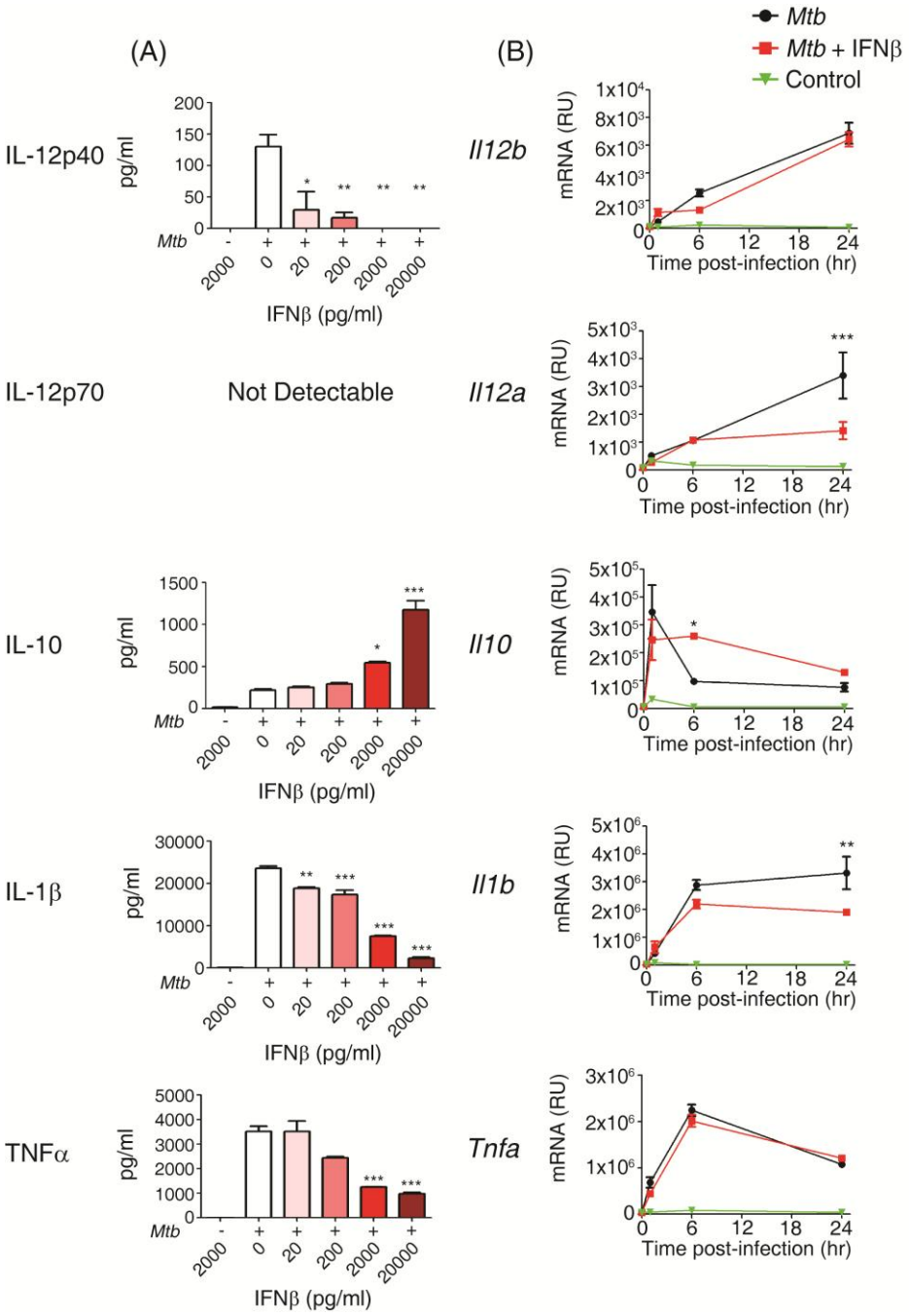

Figure 4.5. Exogenous type I IFN enhances IL-10, and inhibits IL-12, IL-1β and TNFα, from *Mtb* infected macrophages. (A) WT macrophages were infected with *Mtb* in the presence or absence of IFNβ, added concomitantly at the indicated concentration. Cytokine levels in culture supernatant were determined at 24hr post-infection. (B) WT macrophages were infected with *Mtb* in the presence or absence of IFNβ, added concomitantly at 2ng/ml. RNA was harvested at the indicated time points and qPCR analysis was carried out. Graphs show mean +/- SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; one-way ANOVA with Bonferroni post-test vs. *Mtb* only control (part A); two-way ANOVA with Bonferroni post-test (part B). Data is representative of two independent experiments.

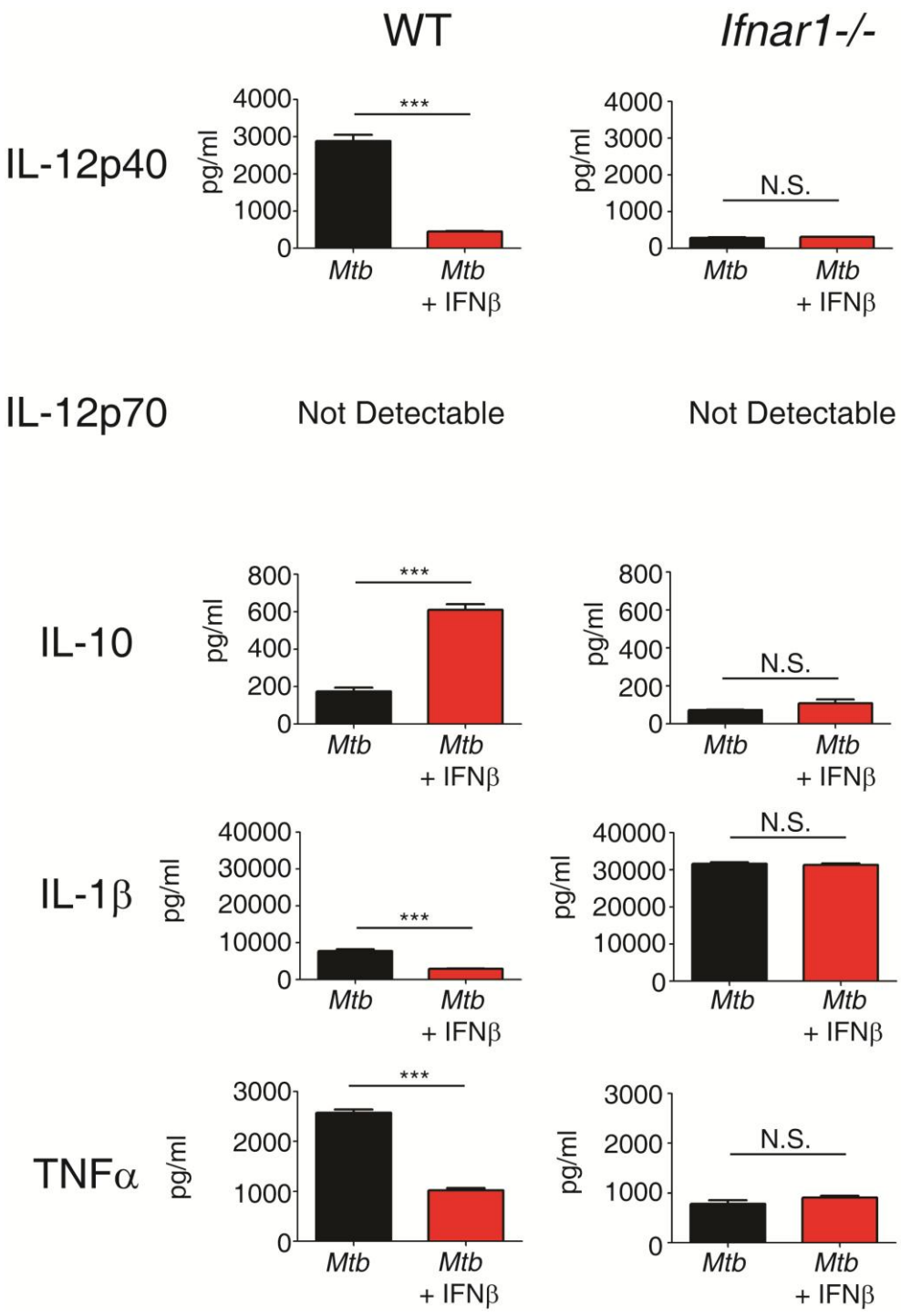

Figure 4.6. IFNβ modulation of cytokine production requires the IFNαβR. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* in the presence or absence of IFNβ, added concomitantly at 2ng/ml. Cytokine levels in culture supernatant were determined at 24hr post-infection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; N.S., not significant; unpaired t-test. Graphs show mean +/- SEM (n=3). Data is representative of three independent experiments.

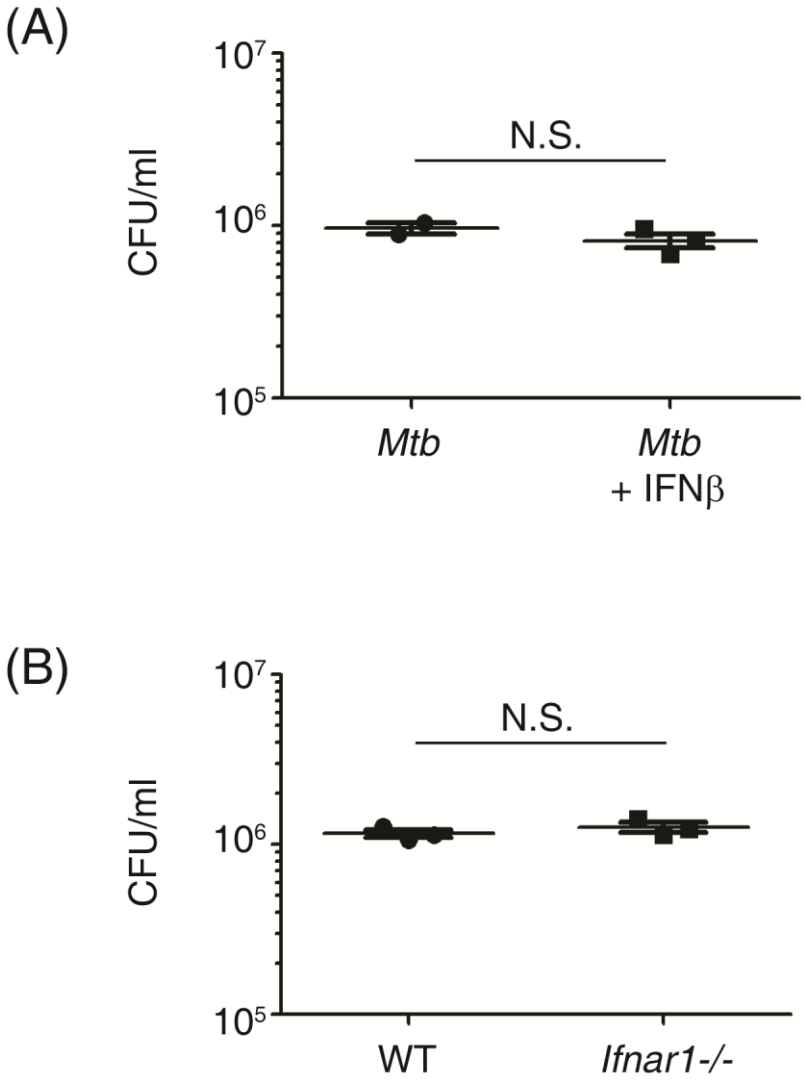

Figure 4.7. Endogenous or exogenous type I IFN does not regulate survival of *Mtb* within macrophages. (A) WT macrophages were infected with *Mtb* in the presence or absence of IFNβ, added concomitantly at 2ng/ml. At 96hr post-infection cell were lysed with 0.2% saponin and bacterial counts were determined by plating onto 7H11 plates supplemented with OADC. (B) WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb*. At 96hr post-infection cell were lysed with 0.2% saponin and bacterial counts were determined by plating onto 7H11 plates supplemented with OADC. N.S.; not significant; unpaired t-test. Data is representative of two independent experiments.

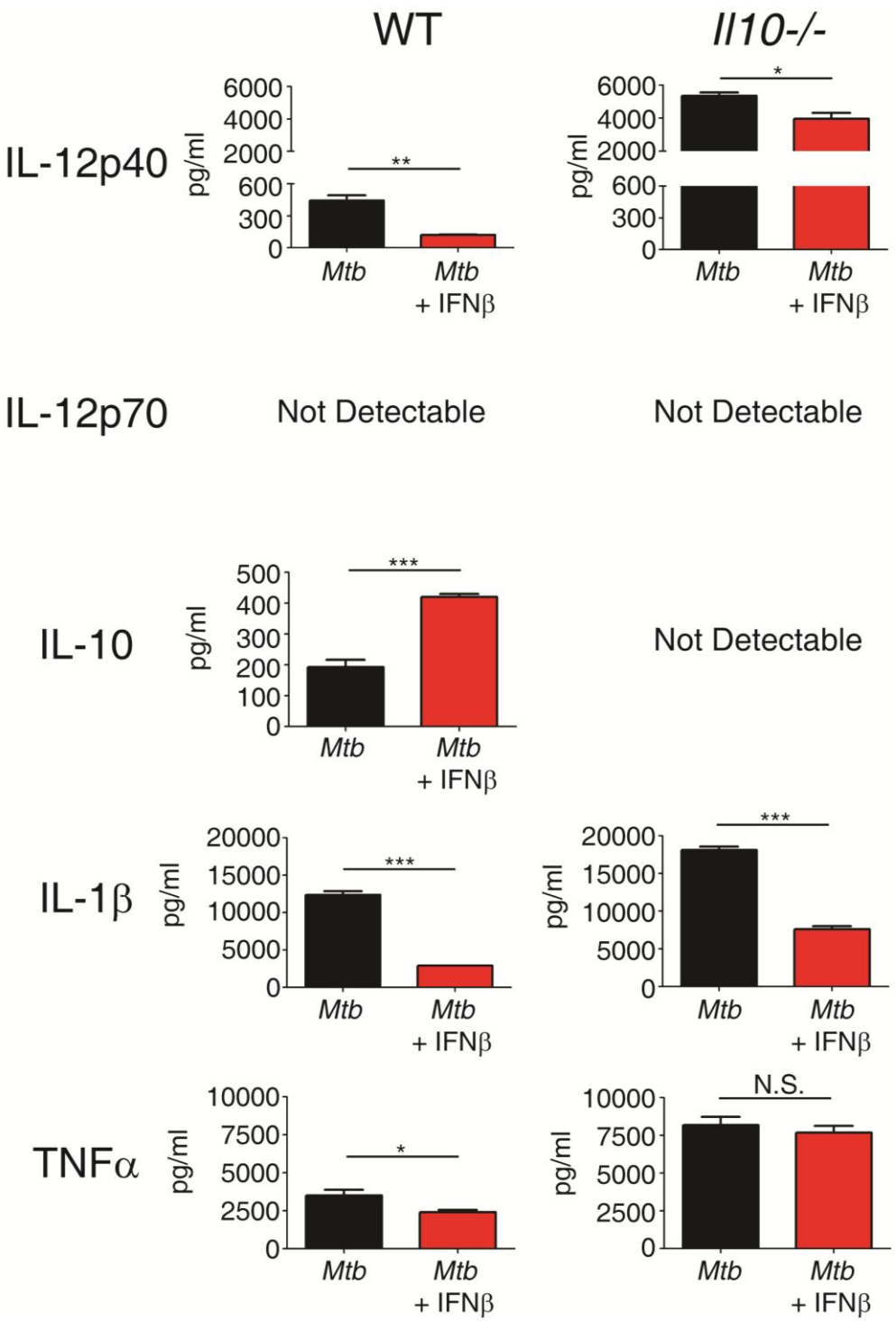

Figure 4.8. IFNβ suppression of IL-12p40 and TNFα is partially dependent on IL-10. WT and *Il10*$^{-/-}$ macrophages were infected with *Mtb* in the presence or absence of IFNβ, added concomitantly at 2ng/ml. Cytokine levels in culture supernatant were determined at 24hr post-infection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, N.S., not significant; unpaired t-test. Graphs show mean +/- SEM (n=3). Data is representative of three independent experiments.

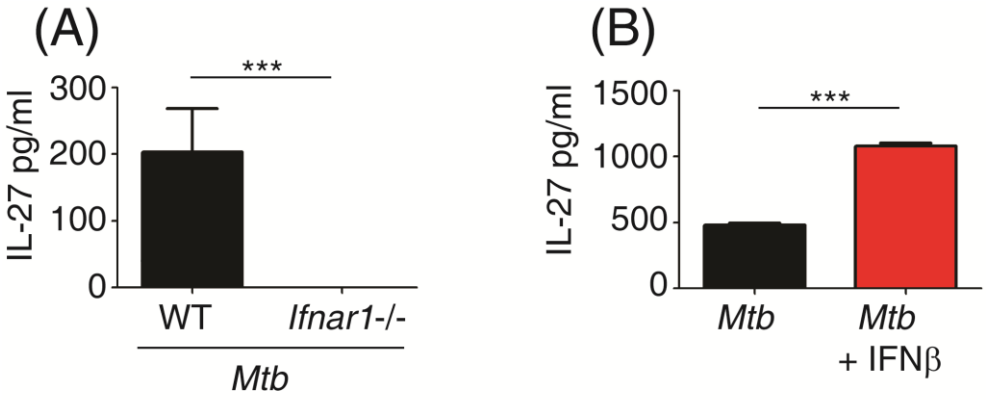

Figure 4.9. Type I IFN promotes IL-27 production from *Mtb*-infected macrophages (A) WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* and levels of IL-27 in culture supernatant were determine by ELISA at 24hr post-infection. (B) WT macrophages were infected with *Mtb* in the presence or absence of 2ng/ml IFNβ, added at the time of infection. Levels of IL-27 in culture supernatant were determined by ELISA at 24hr post-infection. Graphs show mean +/- SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; unpaired t-test. Data is from one experiment.

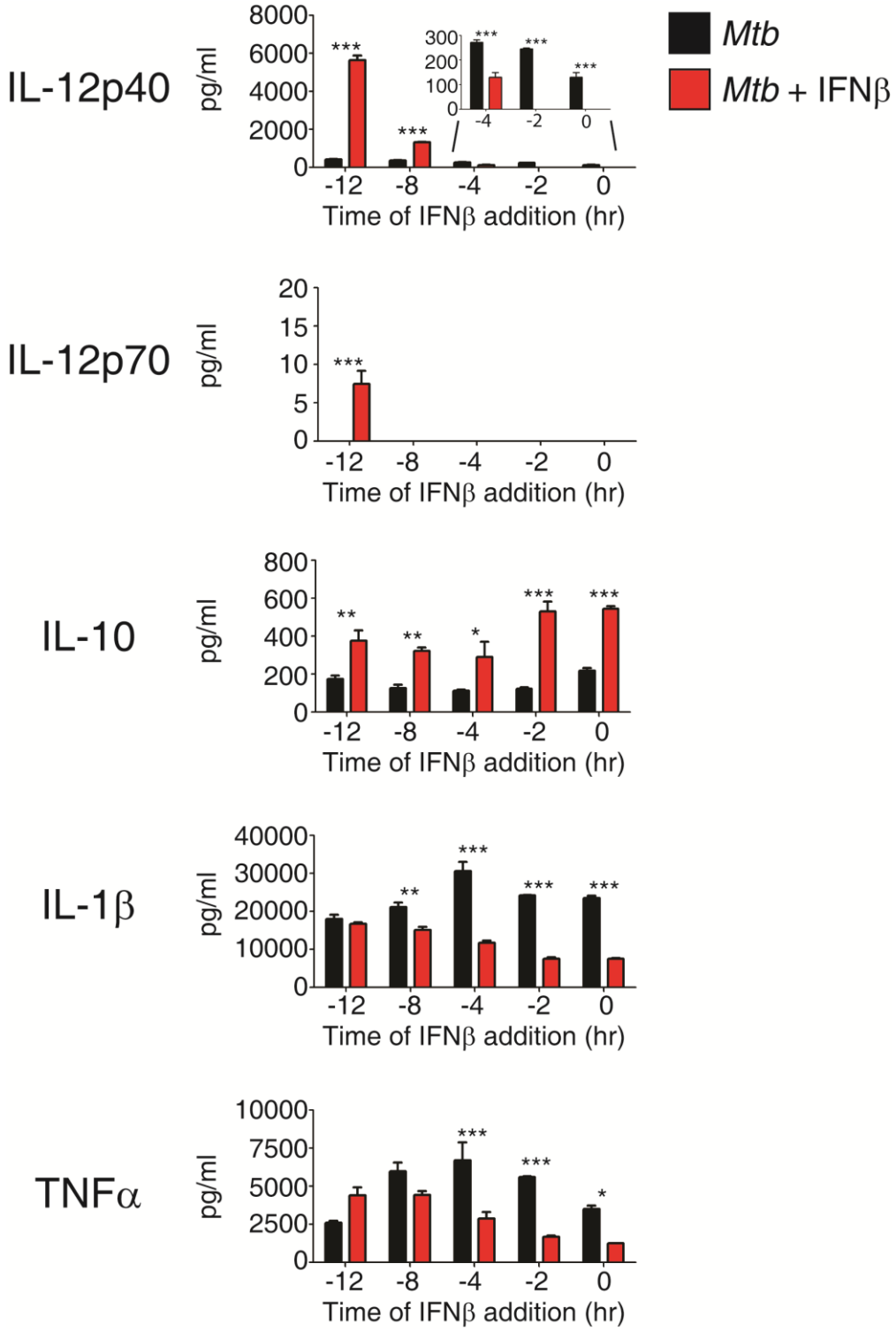

Figure 4.10. IFNβ regulation of IL-12 depends on length of pre-treatment. WT macrophages were infected with *Mtb* in the presence or absence of IFNβ, added at the indicated time prior to infection at 2ng/ml. Cytokine levels in culture supernatant were determined at 24hr post-infection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; two-way ANOVA with Bonferroni post-test. Graphs show mean +/- SEM (n=3). Data is from one experiment.

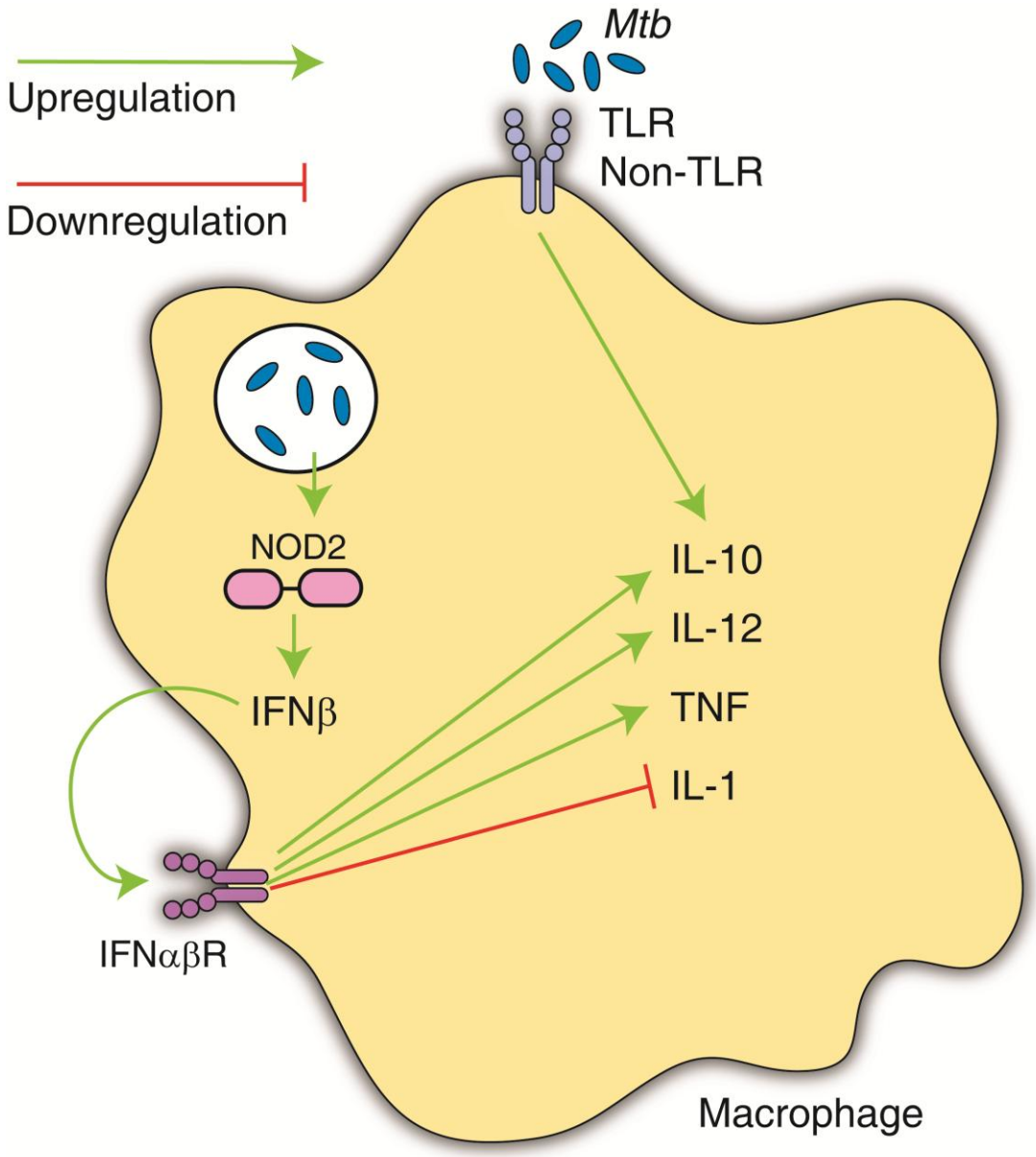

Figure 4.11. A summary of the effects of endogenous type I IFN on cytokine production from *Mtb*-infected macrophages.

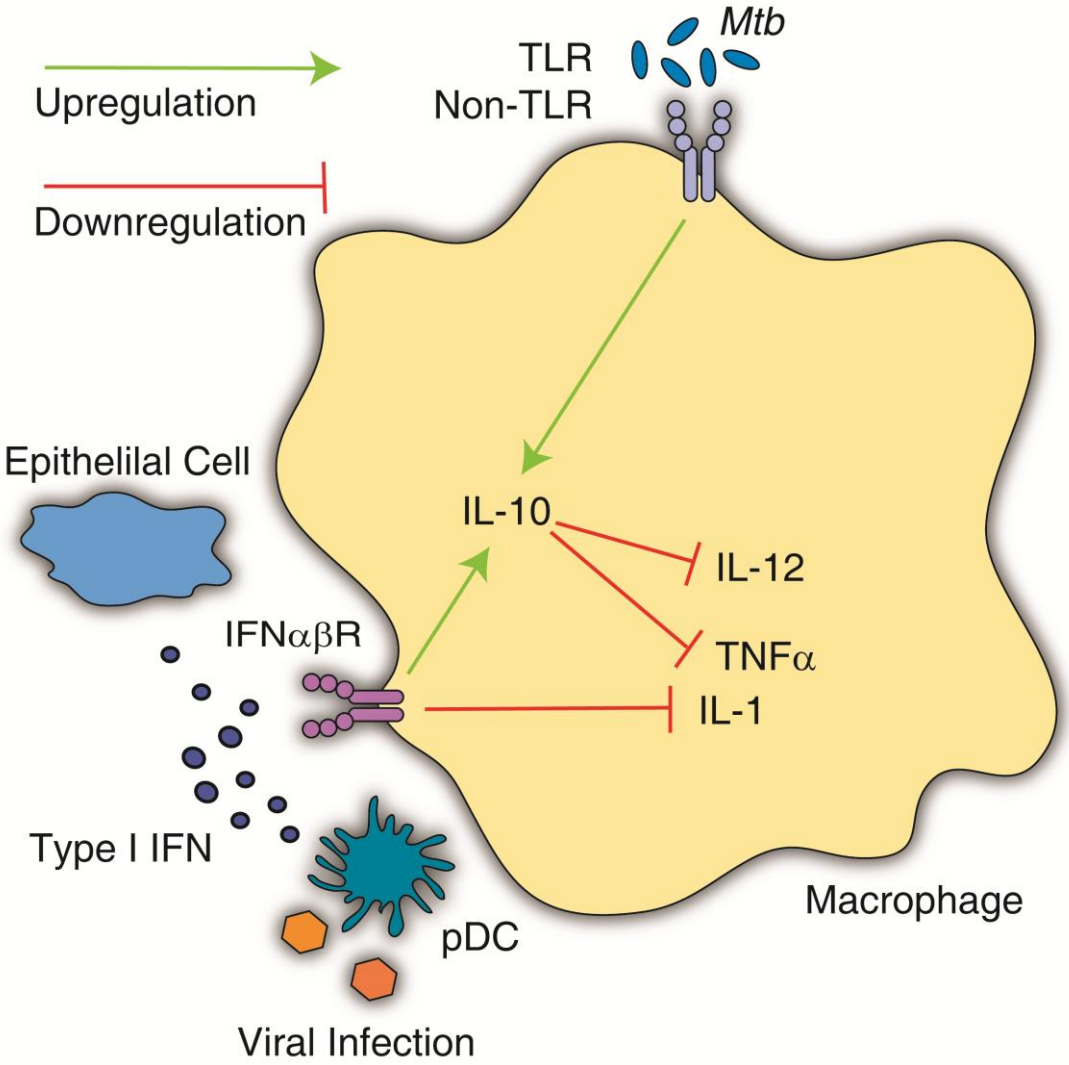

Figure 4.12. The effects of exogenous type I IFN on cytokine production from *Mtb*-infected macrophages.

4.5. Transcripts present in six *k*-means clusters shown in Figure 4.3

Cluster 0

0610009F02Rik	A130072J07	Ccnd2	Hist2h3b
1110008F13Rik	A130072J07Rik	Ccnd2	Hist2h3c1
1110018G07Rik	A430084P05Rik	Cd69	Hmgn3
1110018K11Rik	A430108B07Rik	Cept1	Hmgn3
1110038F14Rik	A530083B17Rik	Ch25h	I830077J02Rik
1110038F14Rik	A630001G21Rik	Chdc1	Ifi205
1110061O04Rik	A630001G21Rik	Cited2	Ifi205
1110061O04Rik	A630077B13Rik	Cited2	IFNg
1110061O04Rik	A930026L03Rik	Cited2	Ifrg15
1200015F23Rik	AA467197	Cox18	Il15
1700001A24Rik	AA589481	Creb5	Il15ra
1700027J05Rik	AA960436	Crkl	Il15ra
2010005J08Rik	AA960436	Crlf3	Il15ra
2010012F05Rik	Adora3	Cspg2	Il15ra
2010012F05Rik	Aff1	Csrp1	Il18
2210019E14Rik	AI837181	Cxcl10	Il2rg
2210019E14Rik	Aim2	Cxcl9	Irf5
2310008I22Rik	Akt3	D11Lgp2e	Katna1
2310020F24Rik	Akt3	D130064H19Rik	Keap1
2610202E01Rik	Aldh1b1	D230004N01Rik	Keap1
2610208M17Rik	Ankrd17	D5Ertd591e	Keap1
2610528H13Rik	Apaf1	D630029K19Rik	Klf4
2700007B13Rik	Apobec3	Dek	LOC215088
2700055A20Rik	Arhgef3	Dll1	LOC223672
2810012G03Rik	Arid5b	Dncic2	LOC227384
2810407C02Rik	Arl4	E330016A19Rik	LOC241293
2810407C02Rik	Asah3	E430021P16Rik	LOC381696
2810474O19Rik	Atp10a	E430034L04Rik	LOC382154
2900002H16Rik	Aytl1	EG240327	LOC383619
4832412D13Rik	Azi2	EG665378	LOC626578
4921505C17Rik	Bambi-ps1	F630035N16Rik	Luzp1
4930422J18Rik	BC003885	Fem1c	Mad
4930453N24Rik	BC016423	Flrt2	Mad
4930555L11Rik	BC038311	Gadd45g	Mcm10
4933412E12Rik	BC049975	Gbp5	Mdk
4933428L19Rik	Bcl9	Gcnt2	Mmp2
4933430F08Rik	Bfar	Gnb4	Mmp25
5430427O19Rik	Bfar	Golga3	Myd88
5730527J01Rik	Bfar	Golga3	Nab1
5730537H01Rik	Birc2	Gpsm2	Nos2
5730589K01Rik	Birc2	Gtpbp2	Ocil
5730596K20Rik	C130045I22Rik	H2-Bf	OTTMUSG00000005523
6230400I06Rik	C330023M02Rik	H3f3b	Parp8
6430402L23Rik	C330023M02Rik	Hat1	Pcaf
7420404O03Rik	C630002C18Rik	Hdc	Pdk3
9130230N09Rik	Card4	Herc1	Peli1
9830137M10Rik	Ccl5	Hist1h3h	Peli1

Cluster 0 Continued	
Pfkp	Tank
Phc2	Tbc1d1
Phca	Tbc1d13
Pi4k2a	Tgtp
Pik3cd	Tlk2
Plekha4	Tlk2
Plekha4	Tlr3
Plekhf2	Tmod3
Plekhf2	Tmpo
Pnp	Tnfrsf1a
Pols	Tnfsf10
Prpf38a	Tnfsf15
Prpf4	Trim21
Prpf4	Trim26
Pttg1	Trim56
Rap2c	Tyk2
Rap2c	Vrk2
Rap2c	Vrk2
Rbl1	Wars
Rfc3	Wars
Rhoe	Wars
Rin2	Wdr20
Rin2	Wdr37
Rnf135	Whdc1
Rnf135	Whsc1l1
Rnf135	Ythdf1
Rnf31	Zbtb5
Sap30	Zcchc6
Sav1	Zfpn1a1
Scarf1	Zyx
scl0001609.1_19	
scl0003154.1_1	
Setdb2	
Sgcb	
Sgk3	
Sgk3	
Slc25a28	
Slc28a2	
Smox	
Snn	
Socs1	
Stat3	
Stxbp3	
Tagap	
Tagap	
Tagap	
Tagap	

Cluster 1

0710001B24Rik	C920008O22Rik	Il15	Ogfrl1
1500032H18Rik	Ccr5	Il21r	P2ry13
1600014C10Rik	Ccr5	Insl6	P2ry14
1600014C10Rik	Cnp1	Irgm	Parp3
1600029O10Rik	Cnp1	Irgm	Pcaf
2310006J04Rik	Cpeb3	Irgm	Phf11
2310016F22Rik	Cutc	Isg20	Pira5
2310046K10Rik	Cutc	Itpr1	Pml
2400003C14Rik	D11Ertd759e	Lgals9	Pml
2410005K17Rik	D11Lgp2e	Lgals9	Pml
2410025L10Rik	D11Lgp2e	LOC209387	Pou3f1
2510004L01Rik	D11Lgp2e	LOC234360	Ppm1k
2810453L12Rik	D14Ertd668e	LOC237751	Prkr
4930566A11Rik	D14Ertd668e	LOC239122	Psmb8
4930599N23Rik	D430007J11Rik	LOC240921	Psmb9
4930599N23Rik	D630022O22Rik	LOC328833	Rab3d
5031414D18Rik	Daxx	LOC380616	Rbm43
5830484A20Rik	Dbnl	LOC380706	Rfc3
6330442E10Rik	Dck	LOC380732	Samhd1
6430573D20Rik	Ddit4	LOC380741	Samhd1
9830148G24Rik	Ddx58	LOC381276	Samhd1
9930016I07Rik	Dlm1-pending	LOC381287	scl000868.1_2
9930111J21Rik	Eng	LOC382127	Scnm1
A230050P20Rik	Enpp4	LOC382177	Scotin
A330042I21Rik	Evi2b	LOC384343	Sertad3
A530023P05Rik	F630107D10Rik	LOC432555	Sirpb1
A530060O05Rik	Fbxo4	LOC434484	Slc28a2
A630067N03Rik	Fcgr1	LOC625360	Slfn1
A630082K20Rik	Frmd4a	MGC6357	Slfn5
A630085K21	G1p2	Mlkl	Sn
Adar	Glipr2	Morc3	Snx2
Aftph	Glipr2	Mov10	Snx2
AI451557	Gpr141	Mov10	Sp100
AI481100	Gvin1	Ms4a6d	Sp100
Aim1	H2-T17	Ms4a6d	St6galnac2
Akap12	H2-T9	Mx1	Stat1
Apaf1	Hars	Mx2	Stat1
Asah2	Hmox2	Nsmaf	Stat1
Asb13	Ifi205	Nsmaf	Stat2
Ass1	Ifi205	Nt5c3	Stat4
AW538212	Ifi47	Oas1a	Symbol
B630009B09Rik	Ifit2	Oas1b	Tap1
BC003314	Ifit2	Oas1b	Tdrd7
BC013712	Ifit3	Oas1g	Timeless
BC023892	Ift172	Oasl1	Tor3a
BC051083	Igtp	Oasl2	Tpst1
BC094916	Il15	Ogfrl1	Trafd1

Cluster 1 Continued
Trafd1
Trex1
Trex1
Trim14
Trim21
Trim30
Tspo
Tspo
Tspo
Tyki
Ube2l6
Vrk1
Zbp1
Zc3hdc1
Zcchc6

Cluster 2

1110003E08Rik	Ctsc	LOC223594	Siat9
1110061O04Rik	Ctsc	LOC230765	Siat9
1110061O04Rik	Ctsc	Loh11cr2a	Skil
1200015F23Rik	Cycs	Lrp11	Slc25a25
1600021P15Rik	D630035N04Rik	Mcoln2	Slc2a1
1810009K13Rik	Dncic2	Mid1	Slfn2
1810038L18Rik	Dusp1	Mlp	Slpi
2210009G21Rik	Dusp2	Mlp	Smg7
2310016C08Rik	Dusp4	Myadm	Snn
2310028H24Rik	Edn1	Nab1	Snx10
4631422O05Rik	Etv3	Nat5	Socs3
4930429A08Rik	F10	Nck1	Spred1
4930555L03Rik	Fmnl2	Ncoa5	Sqstm1
4931440N07Rik	Gadd45b	Nfkbie	Stat3
4933409L06Rik	Gadd45g	Nfkbie	Stx11
4933424M23Rik	Gas7	Npy	Stx11
5730589K01Rik	Gca	Nupr1	Stxbp1
5830457O10Rik	Gca	Pcdh7	Supt16h
6030448M23Rik	Gdf15	Pdcd1lg1	Surb7
8030402P03Rik	Glrx	Pdzgef1	Tank
9130202B12Rik	Glrx1	Pkn2	Tbl1x
9430023P16Rik	Gna15	Plscr1	Tes
9430065L19Rik	Gnb1l	Ppa1	Tlr2
9530018I07Rik	Gpr84	Ppp1r11	Tmem2
A030007L17Rik	Gpr84	Ppp2r5a	Tmpo
A630046C11Rik	Hdac1	Psma4	Tnf
Acate3	Hmgcr	Psmb7	Tnf
AI326906	Hmgcs1	Psmd10	Tra2a
AI427138	Hn1	Ptges	Upp1
Armc8	Hn1l	Rab32	Upp1
AY078069	Hp	Rab5a	Upp1
Aytl1	Hrb	Ranbp1	Vcam1
B230114J08Rik	Hsd17b7	Rars	Vdac3
B430201A12Rik	Ifnar2	Rars	Wrd43
B430201G11Rik	Ikzf1	Rbms1	Zc3h7a
BC004022	Il27	Rela	Zfp281
Brd2	Insig1	Rgl1	Zfp281
Brd2	Irak2	Rgs1	Zfp36
C130032J12Rik	Irg1	Rgs1	Zfpn1a1
Casp7	Irg1	Rgs1	
Ccl2	Jmjd2a	Rps6ka3	
Ccl7	Khdrbs1	Sc4mol	
Ccl7	Kif3c	Sema4a	
Ccrn4l	Klf7	Sertad1	
Cd33	Lcn2	Sfrs10	
Chd1	Ldlr	Sfrs3	
Cln5	LOC194744	Sgk3	

Cluster 3

1110025F24Rik	C030026E19Rik	L3mbtl3	Serpinb6b
1200006F02Rik	C1rl	LOC270152	Slc12a9
1500015G18Rik	C230052I12Rik	LOC383077	Slc8a1
1700009P13Rik	C920006O11Rik	Loh12cr1	Slco2b1
1700012H17Rik	Casp2	Lpxn	St8sia4
1700012H17Rik	Ccnd1	Luc7l2	Stard8
1810020D17Rik	Ccnd1	Maf	Stard8
2400003N08Rik	Ccng2	Mbd5	Tal1
2410015B03Rik	Chd9	Mbnl1	Tcf4
2410081M15Rik	Cpeb3	Mcm10	Thbd
2410193C02Rik	Ctla2b	Mcm6	Thbs1
2610300B10Rik	Cugbp2	Mcm6	Thsd1
2700094F01Rik	Cyp27a1	Mcm6	Tiam1
2810413I22Rik	Cyp27a1	Mcm7	Tlr8
3010026O09Rik	D030012E24Rik	Mef2c	Tm6sf1
4930465J06Rik	D5Ertd585e	Mertk	TNFαip8l2
4930518I15Rik	D5Ertd585e	Mfsd9	TNFαip8l2
4933427G17Rik	Dclre1c	Mier1	Tnfrsf22
4933428I03Rik	Dhrs8	Mppe1	Trim25
5832424M12	Dusp6	Ms4a6b	Txnip
9630007E23Rik	E130102H24Rik	Msrb2	Txnip
9830134K01Rik	Ear10	Msrb2	Uaca
9830139D05Rik	Ehd4	Mtus1	Usf1
A330080J22Rik	Ehd4	Mtus1	Uvrag1
A430105I05Rik	F11r	Ncoa1	Ypel3
A830089I03Rik	F730004D16Rik	Nfatc2ip	Zfp709
Ap3m2	Fgd2	Nqo2	Zfyve26
Apobec1	Fgd4	Nqo2	Zranb3
Arhgap15	Fut10	Olfml2b	
Arhgap18	Gcnt1	Ophn1	
Arhgap25	Gcnt1	P2ry14	
Arl4	Gpr34	P2ry5	
Armc3	Gsg2	Paox	
Atm	Gyg1	Paox	
Atm	Gypc	Plcl2	
AU020772	Hfe	Prkdc	
B230342M21Rik	Hfe	Pscdbp	
Bach2	Hhex	Rab39	
BC003236	Hook2	Rab7l1	
BC003236	Hpse	Rasa3	
BC016201	Ier5l	Rcbtb2	
BC023892	Ift172	Rhobtb1	
Bcl7a	Ift172	Rnase4	
Birc1e	Iqgap2	Rnase6	
Brip1	Kif5c	Rps6ka5	
C030014I23Rik	Klf4	Sca1	
C030025P15Rik	Klra3	scl0015365.1_6	

Cluster 4

1300017K07Rik	Chchd4	Mrps18b	Srm
1700012B18Rik	Clecsf12	Mrps18b	Tbrg4
1700027M21Rik	CRG-L1	Mybbp1a	Tex292
1700051E09Rik	Csrp2	Nfkbil1	Uck2
1810032O08Rik	Cyb5	Nol1	Upf1
2310061F22Rik	D130063H01Rik	Nol11	Zbtb17
2310069P03Rik	D19Bwg1357e	Nol5	Zdhhc12
2310073E15Rik	D8Ertd457e	Npn3	Zfp341
2410131K14Rik	Dnmt3l	Nxn	Srm
2610318C08Rik	Dnmt3l	Panx1	Tbrg4
2810012D02Rik	Dph2	Panx1	Tex292
2810430M08Rik	Dusp3	Pdgfb	Uck2
4732435K05Rik	E430031D18Rik	Pdpk1	Upf1
4930524J08Rik	Ednrb	Pdxp	Zbtb17
4930570C03Rik	Ednrb	Pelo	Zdhhc12
5031409G22Rik	Ednrb	Pim3	Zfp341
5530400B01Rik	F630001K14Rik	Piwil2	
5730436H21Rik	Fkbp11	Pknox1	
5730528L13Rik	Fosl1	Polr3d	
6720458F09Rik	Gfm	Ppan	
8430421H08Rik	Gm1960	Ppapdc1	
9430002A10Rik	Gm22	Ppbp	
9430008C03Rik	Gm237	Ppfibp2	
9930015A14Rik	Gnl3	Ppp3cc	
9930022F21Rik	Gpr68	Ptpre	
A130086G11Rik	Grwd1	Ptrh1	
AA408556	Gss	Rad23b	
AA691260	Heatr3	Rapgef1	
Acat2	Herpud1	Rassf4	
AF322649	Herpud1	Rassf4	
Afg3l2	Hmox1	Rgs3	
AI427809	Hvcn1	Rhob	
Akr1b8	Jmjd3	Rhof	
Alas1	Jund1	Rnu65	
Amotl1	Lad1	Rnu70	
Ampd3	Limd1	Ruvbl2	
Armc7	LOC245350	scl000209.1_5	
AW046014	LOC380625	scl0002445.1_75	
AW046014	LOC381330	Sdccag33	
BC022593	LOC384319	Setd8	
Bhlhb2	Maff	Sf1	
Bmp1	Mdm2	Sla	
C77032	Mettl1	Slc7a11	
C77032	Mftc	Slc7a11	
Cacnb3	Mki67	Slco4a1	
Ccl24	Mki67ip	Srm	
Cdkn2b	Mrps18b	Srm	

Cluster 5

1110001E17Rik	5730403M16Rik	C730026E21Rik	Hyal1
1110032E23Rik	5730547N13Rik	C77080	Inpp5e
1110059F07Rik	6330439P19Rik	C86302	Irs2
1190009E12Rik	6330505F04Rik	C87436	Isca1
1500031N24Rik	6430548M08Rik	Ccnd1	LOC228790
1500041O16Rik	6430548M08Rik	Cd8b	LOC241944
1700027M01Rik	6430567E01Rik	Cdc14b	LOC381297
1700041B20Rik	9130211I03Rik	Centb5	LOC384281
1700052N19Rik	9230102G06Rik	Cerk	Mad2l1bp
1700054N08Rik	9330133O14Rik	Chaf1a	Map3k14
1810010N17Rik	9830001H06Rik	Chchd8	Map3k7ip1
1810011H11Rik	9930031P18Rik	Clspn	Map3k7ip1
1810013C15Rik	A130092J06Rik	Csnk2a1	Mapk1ip1
1810015M01Rik	A230106J09Rik	D330014H01Rik	Mast3
1810045K06Rik	A330103N21Rik	D430036N24Rik	Mbp
1810049H13Rik	A530065E19Rik	D4st1	MGC32441
2010015P12Rik	AA407930	D930048N14Rik	Mlh3
2310003H01Rik	AA408296	Deb1	Ncdn
2310005P05Rik	Aatk	Dedd2	Ncf2
2310036D04Rik	Acot11	Def6	Ndst1
2310042L06Rik	Adcy9	Dnase2a	Ndst1
2310046O06Rik	Adrb2	Dolpp1	Ndst1
2410075B13Rik	AI481316	Dusp7	Nisch
2600005C20Rik	AI842788	E030007N04Rik	Nte
2600005N12Rik	Akt1s1	E430018J23Rik	Nudt6
2610002D18Rik	Ankrd47	Ell	Numa1
2610009I02Rik	Arl4c	Ets1	Nup43
2610029D06Rik	Arrb1	Fahd1	Orc5l
2610206C24Rik	Ase1	Fance	Ormdl3
2610319K07Rik	B230312I18Rik	Fbxl10	P2ry6
2810017I02Rik	B230333C21Rik	Fbxo31	Per1
2810026P18Rik	B3galt4	Fhod1	Per1
3000003F02Rik	B3gnt8	Fignl1	Pex11c
3000003G13Rik	B430214A04Rik	Fpgs	Pgpep1
3110001A13Rik	B430214A04Rik	Fv1	Phf17
3632413B07Rik	BC006705	Galgt1	Pkd1
3632413B07Rik	BC016495	Gemin4	Pla2g4b
3632413B07Rik	BC025546	Gfer	Plekhg3
4430402O11Rik	BC044804	Gig1	Ppgb
4732471D19Rik	BC049806	Gnb5	Ptpn21
4733401I05Rik	BC056929	Gpr146	Rab11fip5
4833408A19Rik	BC060631	Gpr146	Rab3il1
4833425P12Rik	BC061259	Grcc9	Rab40c
4930438D12Rik	Brd3	H13	Rbm38
4930504E06Rik	Brp16	Hpgd	Rbm38
4933402L21Rik	C430004E15Rik	Hps4	Repin1
5630401D24Rik	C530015C18	Hs1bp3	Rgs11

Cluster 5 Continued
Rora
Rpp14
scl0002449.1_77
Sec14l1
Sema4b
Sesn1
Sesn1
Ski
Skp2
Sla
Slc16a6
Slc16a6
Slc19a2
Slc25a38
Slc40a1
Slc5a6
Snapc4
Snx25
Solh
Sox4
Spg20
Strn
Sufu
Surf2
Synj2
Tbc1d10a
Thap11
Tmem143
Tmem20
Tmem24
Tob1
Tprkb
Trim41
Ttll4
Uhrf1
Ung
Wdr22
Wdr77
Zfp251
Zfp27
Zfp422-rs1
Zfp472
Zfp50
Zfp715

Table 4.1.Complete list of genes in the 6 clusters shown in Figure 4.3.

Chapter 5. The TPL2-ERK pathway regulates the macrophage response to *Mtb*, through the suppression of type I IFN

5.1. Background

In response to the recognition of pathogens and pathogen derived products, macrophages initiate an anti-microbial response, and generate inflammation. This occurs through PRRs, which recognise conserved molecular structures of pathogens, and activate signalling pathways leading to transcription (Smale, 2010). The most well characterised PRRs are the TLR family, which activate the Myd88 or TRIF dependent pathways, leading to the activation of NF-κB, MAP kinases and IRFs (Kawai and Akira, 2010). *Mtb* signals through TLRs, predominantly TLR2 and TLR9, but also activates non-TLR pathways including dectin-1 and NOD2 (Kleinnijenhuis *et al*, 2011).

MAP kinase signalling is made up of a three component protein kinase cascade; a MAP 3-kinase, which phosphorylates and activates a MAP 2-kinase, which in turn phosphorylates a MAP kinase (Symons *et al*, 2006). MAP kinases then phosphorylate a wide range of target proteins, including transcription factors. There are three families of MAP kinase; the extracellular signal-regulated kinases (ERK1 and ERK2; referred to subsequently as ERK), the p38 MAP kinases and the c-Jun amino-terminal kinases (JNK1, JNK2 and JNK3) (Symons *et al*, 2006). The first MAP kinase pathway to be discovered, and the most well studied, is the ERK MAP kinase pathway (Gantke *et al*, 2012).

Two MAP 3-kinases are known to lead to ERK activation; members of the Raf family, and TPL2 (Gantke *et al*, 2012). Both kinases phosphorylate and activate the MAP 2-kinases MEK1 and MEK2, which in turn phosphorylate ERK (Gantke *et al*, 2012). TPL2 is required for the activation of MEK1/2 and ERK downstream of TLR signalling, as shown by the fact that macrophages from mice deficient in *Tpl2*

are unable to activate ERK downstream of TLR2, TLR4 and TLR9 ligands (Dumitru *et al*, 2000; Kaiser *et al*, 2009).

The ERK MAP kinase pathway (subsequently referred to as the TPL2-ERK pathway) has been shown to regulate the production of cytokines from TLR stimulated macrophages (Symons *et al*, 2006). Macrophages from mice deficient in *Tpl2* were shown to have impaired production of TNFα in response to LPS (Dumitru *et al*, 2000; Rousseau *et al*, 2008). The effect of the TPL2-ERK pathway on TNFα is thought to be post-transcriptional, as levels of *Tnf* mRNA were the same in WT and *Tpl2* deficient macrophages (Rousseau *et al*, 2008). The TPL2-ERK pathway is also required for optimal production of IL-10 (Agrawal *et al*, 2003; Dillon *et al*, 2004; Kaiser *et al*, 2009) and IL-1β (Mielke *et al*, 2009) in response to various TLR ligands. In contrast, the TPL2-ERK pathway is a negative regulator of IL-12 and IFNβ in macrophages stimulated with LPS and CpG (Kaiser *et al*, 2009; Yang *et al*, 2011). The regulation of IL-10, IL-12 and IFNβ by TPL2-ERK has been shown to depend on the transcription factor c-Fos (Dillon *et al*, 2004; Kaiser *et al*, 2009). However, the mechanism by which TPL2-ERK signalling regulates IL-1β production is unclear.

The cytokines regulated by TPL2-ERK signalling play important roles in the immune response to various pathogens. In general, however, *Tpl2* deficient mice have been shown to be more susceptible to both *Listeria monocytogenes* and *Toxoplasma gondii* (Watford *et al*, 2008; Mielke *et al*, 2009). In the case of *Listeria monocytogenes* infection, this was linked to reduced IL-1β production, whereas increased susceptibility to *Toxoplasma gondii* was suggested to be due to loss of IL-12 and IL-18 induced IFNγ production from $CD4^+$ T cells, showing that TPL2-ERK signalling regulates cytokines from both innate and adaptive immune cells (Watford

et al, 2008; Mielke *et al*, 2009). However, the role of TPL-ERK signalling in *Mtb* infection has not been addressed. Given that TPL2-ERK regulates IL-12, TNFα and IL-1β, all of which are required for protection against *Mtb* (Flynn *et al*, 1995; Cooper *et al*, 1997; Mayer-Barber *et al*, 2011) and IL-10 and type I IFN, which play a detrimental role in the immune response to *Mtb* (Antonelli *et al*, 2010; Redford *et al*, 2010), this pathway would be predicted to play an important role in regulating the immune response to *Mtb*.

As shown in Chapter 3, we identified a cluster of TPL2-ERK inducible genes expressed early in macrophages following *Mtb* infection. This prompted us to investigate the role of the TPL2-ERK pathway in regulating both cytokine production and transcription from macrophages in response to *Mtb*. We found that the TPL2-ERK pathway positively regulated IL-10, IL-1β and TNFα, whilst negatively regulating IL-12 and IFNβ, as has been reported in response to single TLR ligands. The TPL2-ERK pathway also regulated many genes at the transcriptional level. Importantly, several of the changes in cytokine production and transcription in *Tpl2*$^{-/-}$ macrophages were as a result of increased type I IFN signalling.

5.2. Results

5.2.1. The TPL2-ERK pathway regulates induction of early genes in response to *Mtb* infection

Results from microarray analysis presented in Chapter 3 strongly suggested that the TPL2-ERK pathway was rapidly activated in response to *Mtb*. To determine the effect this pathway has on early gene regulation, we compared the response of WT macrophages to *Tpl2*$^{-/-}$ macrophages, which are unable to activate ERK downstream of TLRs (Dumitru *et al*, 2000; Kaiser *et al*, 2009). WT and *Tpl2*$^{-/-}$ macrophages were infected with *Mtb* and at 1hr post-infection RNA was harvested and analysed by microarray.

The microarray data was normalised and undetectable transcripts were removed as described in the Materials and Methods. Transcripts were then retained if they were induced >2-fold in response to *Mtb* in either strain, and if they were significantly differently expressed between WT and *Tpl2*$^{-/-}$ macrophages by two-way ANOVA ($p<0.05$) with Benjamini Hochberg FDR multiple testing correction. This left 422 transcripts which were clustered hierarchically (Figure 5.1A).

The majority of the 422 transcripts were regulated in the uninfected controls at 1hr post-infection (Figure 5.1A), possibly reflecting a role for *Tpl2* in the macrophage response to growth factors present in the culture medium. However, a small cluster of 24 *Tpl2*-dependent *Mtb*-regulated transcripts was identified (Figure 5.1B). This cluster included several genes present in cluster 15 of the microarray time-course, including *Fos*, *Egr1*, *Dusp1* and *Dusp5* (Figure 5.1B). In addition, a number of other genes were found to be regulated by the TPL2-ERK pathway. These included the chemokines *Ccl2*, *Ccl4* and *Ccl7*, immunoresponsive gene 1 (*Irg1*) and the actin related gene *Arc*, which has previously been linked to ERK signalling

(Huang *et al*, 2007) (Figure 5.1B). The reduced expression of *Fos* and *Egr1* in *Tpl2*$^{-/-}$ macrophages at 1hr post-infection was confirmed by qPCR analysis (Figure 5.1C). The TPL2-ERK pathway is therefore an important regulator of early transcription in *Mtb*-infected macrophages.

5.2.2. The TPL2-ERK pathway negatively regulates IL-12 and IFNβ, but positively regulates IL-10, IL-1β and TNFα from *Mtb* infected macrophages

The TPL2-ERK pathway has previously been shown to be a key regulator of cytokine production in innate immune cells, in response to a variety of TLR ligands, including LPS, CpG and Pam3CSK4 (Dumitru *et al*, 2000; Dillon *et al*, 2004; Kaiser *et al*, 2009; Mielke *et al*, 2009). This included several cytokines that have important effects in the immune response to *Mtb* including TNFα, IL-1β and IL-12, IL-10 and IFNβ. The regulation of cytokine production by TPL2-ERK signalling has been reported to be due to the activation of the transcription factor c-Fos (Dillon *et al*, 2004; Kaiser *et al*, 2009), which we found was dependent on TPL2-ERK for its induction in response to *Mtb* (Figure 5.1). We therefore determined if the TPL2-ERK pathway also regulated cytokine production in macrophages in response to *Mtb*, which signals through both TLR and non-TLR receptors (Kleinnijenhuis *et al*, 2011).

WT and *Tpl2*$^{-/-}$ macrophages were infected with *Mtb* and cytokine production determined at 24hr by ELISA. In comparison to WT macrophages, *Tpl2*$^{-/-}$ macrophages produced markedly increased levels of both IL-12p40 and IL-12p70 in response to *Mtb* (Figure 5.2A). In addition, levels of IFNβ were also higher in *Tpl2*$^{-/-}$ macrophages (Figure 5.2A). The TPL2-ERK pathway therefore negatively regulates IL-12 and IFNβ production from *Mtb* infected macrophages. In contrast, levels of

IL-10, IL-1β and TNFα were reduced in *Tpl2*$^{-/-}$ macrophages, showing a positive effect of the TPL2-ERK pathway on the production of these cytokines (Figure 5.2A).

To confirm these findings, a second independent approach was used to disrupt the TPL2-ERK pathway. This was important for two reasons; first that TPL2 may have other phosphorylation targets besides MEK1/2 (although this has not yet been proven) (Gantke *et al*, 2011) and second because TPL2 is associated with the IKK family member p105 in steady state, and thus loss of *Tpl2* may have effects on NF-κB signalling (Gantke *et al*, 2012). To address this, we inhibited TPL2-ERK signalling downstream of TPL2, using PD0325901, a highly specific inhibitor of MEK1/2 kinase activity. PD0325901, or DMSO as a vehicle control, was added to WT macrophages 30mins prior to *Mtb* infection, and cytokine production was determined at 24hr post-infection (Figure 5.2B). Results using the MEK1/2 inhibitor closely resembled the phenotype of *Tpl2*$^{-/-}$ macrophages, with increased levels of IL-12p40, IL-12p70 and IFNβ, but decreased levels of IL-10, IL-1β and TNFα, compared to macrophages treated with DMSO vehicle control (Figure 5.2B). This was also confirmed in primary *Mtb* infected monocytes from WT and *Tpl2*$^{-/-}$ mice (McNab, O'Garra; data not shown). These results are in keeping with previous studies using single TLR ligands (Dumitru *et al*, 2000; Dillon *et al*, 2004; Kaiser *et al*, 2009; Mielke *et al*, 2009) and confirm that the TPL2-ERK pathway is an important regulator of cytokine production from *Mtb* infected macrophages.

5.2.3. TPL2 negatively regulates IL-12 production independently of IL-10

Interruption of the TPL2-ERK pathway leads to reduced IL-10 but increased IL-12 production (Figure 5.2). It was possible that the increased production of IL-12

observed was a result of lower IL-10 levels, as IL-10 is a potent inhibitor of IL-12 from APCs (D'Andrea *et al*, 1993; Moore *et al*, 2001). To address this, *Tpl2*$^{-/-}$ mice were crossed with *Il10*$^{-/-}$ mice to produce *Tpl2*$^{-/-}$*Il10*$^{-/-}$ mice. Macrophages from *Tpl2*$^{-/-}$*Il10*$^{-/-}$ mice, and WT, *Il10*$^{-/-}$ and *Tpl2*$^{-/-}$ macrophages, were infected with *Mtb* and the production of IL-12p40 and IL-12p70 was determined at 24hr post-infection. The crucial comparison in this experiment is between *Il10*$^{-/-}$ macrophages and *Tpl2*$^{-/-}$*Il10*$^{-/-}$ macrophages, as this shows the effect of removing TPL2-ERK signalling in the absence of endogenous IL-10. As shown in Figure 5.3, a significant increase in both IL-12p40 and IL-12p70 production was observed in *Tpl2*$^{-/-}$*Il10*$^{-/-}$ macrophages compared to *Il10*$^{-/-}$ macrophages. This increase was to a similar level than the increase observed between WT and *Tpl2*$^{-/-}$ macrophages (Figure 5.3). This shows that TPL2-ERK signalling regulates the production of IL-12 independently of IL-10.

5.2.4. The TPL2-ERK pathway is an important regulator of transcription in *Mtb* infected macrophages

Previous results demonstrate that the TPL2-ERK pathway regulates both the early macrophage response to *Mtb* and the production of cytokines. As a number of transcription factors were part of the early TPL2-ERK dependent response, we next assessed what impact this may have on subsequent gene expression at a later time point. In addition, this would reveal whether the regulation of cytokines by TPL2-ERK signalling is transcriptional or post-transcriptional. To address this we carried out microarray analysis of WT and *Tpl2*$^{-/-}$ macrophages infected with *Mtb* at 6hr post-infection, the peak of the transcriptional response to *Mtb* (see Chapter 3). The data were normalised and undetectable transcripts were removed, as described in the Materials and Methods. Differentially regulated transcripts were generated by

retaining those that were >2-fold upregulated by *Mtb* in either WT or *Tpl2*$^{-/-}$ macrophages, those that were significantly different between WT and *Tpl2*$^{-/-}$ by two-way ANOVA with Benjamini Hochberg FDR multiple testing correction, and those that were greater than 2-fold different between WT and *Tpl2*$^{-/-}$ macrophages infected with *Mtb* at 6hr post-infection. With this analysis strategy, 104 *Mtb*-inducible transcripts were found to be differentially regulated between WT and *Tpl2*$^{-/-}$ macrophages at 6hr post-infection (Figure 5.4A).

A prominent cluster was increased to a much greater extent in *Tpl2*$^{-/-}$ macrophages (Figure 5.4A). This included *Il12a*, which encodes for the p35 subunit of IL-12, in keeping with the fact that IL-12p70 is upregulated in *Tpl2*$^{-/-}$ macrophages at the protein level (Figure 5.2A). In addition, several subtypes of IFNα were in this cluster, including *Ifna2*, *Ifna5* and *Ifna6*, showing that TPL2-ERK can regulate several members of the type I IFN family. The increased expression of *Il12a*, *Ifna2*, *Ifna5* and *Ifna6* in *Tpl2*$^{-/-}$ macrophages was validated by qPCR (Figure 5.4B). This cluster also included *C2ta*, which codes for the Class II transactivator, a master regulator of components of the MHC class II pathway (Boehm *et al*, 1997). This shows that a number of immunologically important genes are negatively regulated by TPL2-ERK signalling. In addition, several genes with more diverse functions were increased in *Tpl2*$^{-/-}$ macrophages. This included two genes encoding calcium ion channels; *Clic5* (chloride intracellular ion channel 5) and *Cacnb3* (calcium channel, voltage-dependent, beta 3 subunit). In addition, two genes involved in chromatin remodelling were upregulated in *Tpl2*$^{-/-}$ macrophages; *Hmgn3*, involved in histone acetylation (Barkess *et al*, 2012), and *Jmjd2a* (KDM4A), a histone demethylase (Couture *et al*, 2007). This suggests that TPL2-ERK may

regulate histone modifications, which may have an important effect on gene regulation.

A second prominent cluster of transcripts was downregulated in response to *Mtb*, but which were downregulated to a greater extent in *Tpl2*$^{-/-}$ macrophages (Figure 5.4A). TPL2-ERK signalling therefore regulates both the induction and suppression of transcription in *Mtb*-infected macrophages. Genes in this cluster included a subunit of the IFNγ receptor (*Ifngr1*) and the enzyme arginase (*Arg1*) (Figure 5.4A). Reduced expression of *Ifngr1* in *Tpl2*$^{-/-}$ macrophages was confirmed by qPCR (Figure 5.4B). The stimulation of macrophages by IFNγ is vital for controlling *Mtb* infection, as this leads to the killing of intracellular *Mtb*. The downregulation of the *Ifngr1* in the absence of TPL2-ERK signalling may therefore impair the host immune response to *Mtb*.

The 104 differentially regulated transcripts did not contain IFNβ, IL-1β, TNFα or IL-10, despite differences in these cytokines being observed at the protein level in *Tpl2*$^{-/-}$ macrophages compared to WT (Figure 5.2A). To determine if these genes were differentially regulated at the transcriptional level, qPCR analysis was carried out. In keeping with the protein data, levels of *Il10* and *Il1b* were significantly lower in *Tpl2*$^{-/-}$ macrophages, compared to WT, and IFNβ was significantly upregulated (Figure 5.4B). However, no difference was observed for *Tnf*, suggesting that TNFα is entirely regulated at the post-transcriptional level by the TPL2-ERK pathway, as previously reported (Dumitru *et al*, 2000; Rousseau *et al*, 2008).

5.2.5. The TPL2-ERK pathway positively regulates IL-1β, and negatively regulates IL-12p70, through the suppression of type I IFN

Following microarray analysis, it was clear that a prominent function of the TPL2-ERK signalling pathway is to negatively regulate type I IFN production, as in the absence of this pathway several members of the type I IFN family are increased, including IFNβ and three subtypes of IFNα (Figure 5.2; Figure 5.4). The results from Chapter 3 showed that endogenous type I IFN was an important regulator of both cytokine production and transcription in *Mtb* infected macrophages. It was therefore possible that some of the effects observed in the absence of TPL2-ERK signalling could be a result of increased type I IFN.

In order to investigate the contribution of type I IFN to the phenotype of *Tpl2*$^{-/-}$ macrophages, *Tpl2*$^{-/-}$ mice were crossed with *Ifnar1*$^{-/-}$ mice to produce *Tpl2*$^{-/-}$ *Ifnar1*$^{-/-}$ mice. Thus, comparison of *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ mice to *Ifnar1*$^{-/-}$ mice would show the effect of blocking TPL2-ERK in the absence of type I IFN signalling. Macrophages from these mice and respective controls were then infected with *Mtb* and cytokine production was determined at 24hr post-infection.

As shown previously, *Tpl2*$^{-/-}$ macrophages produced increased amounts of IL-12p40 compared to WT, whereas *Ifnar1*$^{-/-}$ macrophages produced less (Figure 5.5). However, *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ macrophages produced higher levels than *Ifnar1*$^{-/-}$ macrophages (Figure 5.5). In both cases the increase was approximately 10-fold, showing that the increase in IL-12p40 production in the absence of TPL2 is independent of type I IFN, despite overall IL-12p40 being dependent upon type I IFN. However, although levels of IL-12p70 increased in *Tpl2*$^{-/-}$ macrophages, the same increase was not seen in *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ macrophages (Figure 5.5) showing that

the increased type I IFN observed in *Tpl2*$^{-/-}$ macrophages drives increased IL-12p70 production.

Both TPL2 and type I IFN signalling are positive regulators of IL-10, and so IL-10 levels were lower in both *Tpl2*$^{-/-}$ and *Ifnar1*$^{-/-}$ macrophages, and lower again in *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ (Figure 5.5). TPL2-ERK and type I IFN therefore regulate IL-10 independently. A similar pattern was seen for TNFα production, as both TPL2 and type I IFN signalling are required for maximal production of TNFα (Figure 5.5).

However, the decrease in IL-1β production seen in *Tpl2*$^{-/-}$ macrophages was not observed in the absence of type I IFN signalling; *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ macrophages had comparable levels of IL-1β to *Ifnar1*$^{-/-}$ macrophages (Figure 5.5). The TPL2-ERK pathway therefore positively regulates IL-1β indirectly, through the suppression of type I IFN production. The increase in IFNβ production in *Tpl2*$^{-/-}$ macrophages is relatively small (Figure 5.2). However, previous results have shown that very low levels of exogenous IFNβ (20pg/ml) could inhibit IL-1β (see Chapter 4) showing that IL-1β is highly sensitive to suppression by type I IFN.

5.2.6. The TPL2-ERK pathway regulates transcription by suppressing type I IFN production

As TPL2-ERK regulates IL-1β and IL-12p70 production indirectly through type I IFN, it was also possible that increased type I IFN production was responsible for the broader transcriptional changes observed in *Tpl2*$^{-/-}$ macrophages. To investigate this, microarray analysis was carried out using WT, *Tpl2*$^{-/-}$, *Ifnar1*$^{-/-}$ and *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ mice infected for 6hr with *Mtb*. Following normalisation, the expression of the 104 *Tpl2*-regulated transcripts shown in Figure 5.5 was determined.

Many of the genes up or downregulated in *Tpl2*$^{-/-}$ macrophages are regulated in a type I IFN dependent manner. A prominent cluster of genes, including *Ifna2, Ifna5, Ifna6* and *Il12a* were not upregulated in *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ macrophages relative to *Ifnar1*$^{-/-}$, despite being upregulated in *Tpl2*$^{-/-}$ versus WT macrophages (Figure 5.6A). This data with *Il12a* is in keeping with the type I IFN dependent increase in IL-12p70 at the protein level (Figure 5.5). The expression of *Ifna2*, *Ifna5, Ifna6* and *Il12a* was validated by qPCR (Figure 5.6B). Other genes regulated in a type I IFN dependent manner included the TAP-related gene *Tapbpl* (TAP binding protein-like), the chloride ion channel *Clic5* and the GTP-activating *Rgs3* (Figure 5.6A). This shows that many of the effects of TPL2-ERK on transcriptional regulation occur indirectly, through autocrine type I IFN.

In addition, a further cluster was downregulated in *Tpl2*$^{-/-}$ macrophages in a type I IFN dependent manner (Figure 5.6A). This included a subunit of the receptor for IFNγ, *Ifngr1*, expression of which was validated by qPCR (Figure 5.6B). Expression of the IFNγ receptor in macrophages is crucial during *Mtb* infection, as IFNγ stimulates macrophages to kill *Mtb*. Other genes in this cluster included the enzyme *Arg1* (encoding for arginase) and *Dusp6* (Figure 5.6A).

5.3. Discussion

The response of macrophages to pathogens and pathogen-derived components is critical in protecting the host from infection. The signalling pathways that regulate this response downstream of PRRs are therefore of great interest. We show here that the TPL2-ERK MAP kinase signalling pathway is an important regulator of the macrophage response to *Mtb*. TPL2-ERK signalling regulated both early and late transcription in *Mtb*-infected macrophages. In addition, TPL2-ERK regulated the production of cytokines vital for protection against *Mtb*; TNFα, IL-1β and IL-12 (Cooper *et al*, 1995; Flynn *et al*, 1995; Mayer-Barber *et al*, 2011) and cytokines known to reduce resistance to *Mtb*; IL-10 and IFNβ (Antonelli *et al*, 2010; Redford *et al*, 2010).

5.3.1. The regulation of cytokine production by the TPL2-ERK pathway

We found that the TPL2-ERK pathway regulated a number of important cytokines in response to *Mtb*. TPL2-ERK positively regulated IL-10, IL-1β and TNFα in response to *Mtb*, but negatively regulated IL-12p40, IL-12p70 and IFNβ. A summary of the effects of TPL2-ERK signalling on macrophage cytokine production in response to *Mtb* is shown in Figure 5.7.

These results are largely in agreement with previous studies with macrophages treated with single TLR ligands such as LPS, CpG and Pam3CKS4 (Dumitru *et al*, 2000; Agrawal *et al*, 2003; Dillon *et al*, 2004; Rousseau *et al*, 2008; Kaiser *et al*, 2009; Mielke *et al*, 2009; Yang *et al*, 2011). One study has reported conflicting results; Xiao *et al* (2009) found that a mutation in *Tpl2* resulted in impaired type I IFN production from peritoneal macrophages stimulated with TLR7

and TLR9 agonists. However, this study used mice in which *Tpl2* was mutated using *N*-ethyl-*N*-nitrosourea (Xiao *et al*, 2009), a process which can result in only partial loss of function, as opposed to the complete loss of function in *Tpl2*$^{-/-}$ mice.

The transcription factor c-Fos, which is part of the early cluster induced by *Mtb* in a TPL2-ERK dependent manner, has previously been shown to promote IL-10 production, and inhibit IL-12 and IFNβ, downstream of TPL2-ERK signalling (Dillon *et al*, 2004; Kaiser *et al*, 2009). Dillon *et al* (2004) showed that CD11c^{+} DCs from c-Fos deficient mice had reduced IL-10 production in response to LPS and Pam3CSK4. Subsequently, it was shown that retroviral expression of c-Fos in *Tpl2*$^{-/-}$ DCs was sufficient to inhibit IL-12p40, IL-12p70 and IFNβ production in response to CpG (Kaiser *et al*, 2009). We also observed that induction of *Fos* at the mRNA level was impaired in *Tpl2*$^{-/-}$ macrophages, suggesting that the effects seen in *Tpl2*$^{-/-}$ macrophages may be due to a loss of c-Fos activity. Further experiments using mice deficient in *Fos* could address this.

We show here that the important pro-inflammatory cytokine IL-1β is dependent upon TPL2-ERK signalling for its expression in macrophages infected with *Mtb*. This is in agreement with a previous study using TLR ligands such as LPS and Poly-ICLC (Mielke *et al*, 2009). In agreement with our study, Mielke *et al* (2009) showed that mRNA levels of pro-IL-1β were lower in *Tpl2*$^{-/-}$ macrophages, suggesting regulation at the transcriptional level. However, this study did not address the mechanism behind the regulation of IL-1 by TPL2-ERK signalling. Our study found that TPL2-ERK signalling positively regulates IL-1β production indirectly, via the negative regulation of type I IFN. Type I IFN is a potent suppressor of IL-1β production, and so if the TPL2-ERK pathway is blocked, levels of type I IFN increase and feed back to inhibit IL-1β. This was demonstrated by the fact that no

loss of IL-1β was observed in *Tpl2*$^{-/-}$ macrophages if type I IFN signalling was also removed; *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ macrophages had comparable levels to *Ifnar1*$^{-/-}$ controls.

The results presented here are limited to the study of bone marrow derived macrophages. Although macrophages are an important part of the innate immune response to *Mtb*, many other innate immune cells are infected, and produce cytokines, during infection (Wolf *et al*, 2007). Importantly, others in our laboratory have shown that monocytes taken directly *ex vivo* from the blood of WT and *Tpl2*$^{-/-}$ mice showed similar effects of TPL2-ERK signalling, with reduced IL-10, IL-1β and TNFα, and enhanced IL-12 and IFNβ, in *Tpl2*$^{-/-}$ monocytes (McNab, Ewbank, Stavropoulos, O'Garra; Manuscript submitted). This shows that TPL2-ERK signalling is likely to play an important role *in vivo*. Further work could address this effect in additional cell types. For example, myeloid DCs are reported to be the major producers of IL-12p70 *in vivo* during *Mtb* infection (Rothfuchs *et al*, 2009) and so the role of TPL2-ERK signalling in these cells could be of interest. In addition, it would be important to address the role of TPL2-ERK signalling in alveolar macrophages, as these are thought to be the first cells to encounter *Mtb in vivo* (Cooper, 2009).

5.3.2. Wider transcriptional regulation by TPL2-ERK signalling

The TPL2-ERK pathway is clearly an important regulator of cytokine production in response to *Mtb* infection in macrophages. However, microarray analysis in this study shows that TPL2-ERK signalling regulates many genes at the transcriptional level. This included both genes induced early following *Mtb* infection (at 1hr post-infection) and those induced later (6hr post-infection). Regulation of

these genes by TPL2-ERK may have important consequences in the macrophage response to *Mtb*.

A number of early genes were regulated by TPL2-ERK signalling. This included the transcription factor c-Fos, which is likely to be involved in regulating cytokine production, as discussed above. The zinc-finger transcription factor *Egr1* was also found to be dependent upon TPL2-ERK for its expression downstream of *Mtb*. *Egr1* is induced in response to many stimuli, and has been linked with a number of functions including growth control and apoptosis (Thiel and Cibelli, 2002). Previous studies have shown that both enteropathogenic *E.coli* and *Chlamydia pneumoniae* induce *Egr1* expression in HELA cells and RAW macrophages respectively (de Grado *et al*, 2001; Bea *et al*, 2003). In agreement with our results, the induction of *Egr1* was in both cases found to depend on ERK signalling, as inhibition of MEK1/2 activity prevented *Egr1* induction (de Grado *et al*, 2001; Bea *et al*, 2003). That *Egr1* is induced during a number of different infections suggests it may be playing an important role in the immune response.

Loss of TPL2-ERK signalling also regulated many genes at 6hr post-infection. However, whereas TPL2-ERK was mainly a positive regulator of transcription at 1hr, it appeared to be mainly a negative regulator at 6hr post-infection, with the majority of genes showing increased expression in *Tpl2*$^{-/-}$ macrophages. A number of these changes were due to increased levels of type I IFN signalling, as increases were not seen in *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ macrophages relative to *Ifnar1*$^{-/-}$ controls. This included several subsets of IFNα; *Ifna2*, *Ifna5* and *Ifna6*. This is in keeping with the fact that expression of IFNα genes is known to depend on autocrine signalling through IFNβ, in a positive feedback loop involving IRF-7 (Marie *et al*, 1998; Sato *et al*, 1998). Increased levels of IFNβ in *Tpl2*$^{-/-}$

macrophages, which are observed at the protein level, therefore feed back to drive increased amounts of IFNα.

5.3.3. The role of TPL2-ERK signalling in the *in vivo* response to *Mtb*

Our results show that TPL2-ERK signalling regulates a number of cytokines known to have important effects in the immune response to *Mtb*. However, whether loss of TPL2-ERK signalling would have a beneficial or detrimental effect on the host response to *Mtb* is unclear. The positive regulation of TNFα and IL-1β by TPL2-ERK signalling would be expected to protect the host from *Mtb*, as both these cytokines are known to be required for protection (Flynn and Chan, 2001a; Mayer-Barber *et al*, 2011). In addition, as type I IFN is thought to play a negative role in infection, the suppression of type I IFN by TPL2-ERK would also be predicted to benefit the host. In contrast, TPL2-ERK signalling positively regulates IL-10, known to suppress the immune response to *Mtb* (Redford *et al*, 2010) and negatively regulates IL-12, which is required for the protective Th1 response (Cooper, 2009). TPL2-ERK could therefore have both positive and negative effects on the response to *Mtb*.

However, *in vivo* studies from our laboratory (carried out by F.McNab and E.Stavropoulos) have shown that *Tpl2*$^{-/-}$ mice are more susceptible to *Mtb* infection than WT controls (McNab, Ewbank, Stavropoulos, O'Garra; manuscript submitted). *Tpl2*$^{-/-}$ mice had around a 1-log increase in bacterial burden in the lungs and spleen following infection. Further investigation showed that the key reason for this increased susceptibility was increased type I IFN production, as the effect could be prevented by blocking type I IFN signalling; *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ mice were no more

susceptible than *Ifnar1*$^{-/-}$ controls (McNab, Ewbank, Stavropoulos O'Garra; manuscript submitted). This is in agreement with the *in vitro* results presented in this chapter, as many of the effects of blocking TPL2-ERK signalling, including the loss of IL-1β and changes in transcription, were due to an increase in type I IFN signalling.

Tpl2$^{-/-}$ mice were also more susceptible to infection with *Listeria monocytogenes*, with *Tpl2*$^{-/-}$ mice showing increased bacterial burdens in the spleen. Again, this effect was dependent upon type I IFN (McNab, Ewbank, Stavropoulos O'Garra; manuscript submitted). TPL2-ERK signalling therefore protects the host from infection with both *Mtb* and *Listeria* through the suppression of type I IFN. Further investigation into the mechanism behind this effect found that increased type I IFN production suppressed the Th1 response in *Tpl2*$^{-/-}$ mice. In contrast to results *in vitro*, *Tpl2*$^{-/-}$ mice showed no increase in IL-12 production in the serum following infection with *Listeria monocytogenes* (McNab, Ewbank, Stavropoulos, O'Garra, manuscript submitted). This could be reversed, however, by removing type I IFN signalling, as *Tpl2*$^{-/-}$*Ifnar1*$^{-/-}$ mice showed dramatically increased IL-12p70 in the serum, and an increase in the Th1 cytokine IFNγ. This is in keeping with our *in vitro* results showing addition of exogenous IFNβ can suppress IL-12 production, and suggests that *in vivo*, high levels of type I IFN can suppress the Th1 response.

Due to its role in the induction of TNFα, there is considerable interest in developing drugs that target TPL2, as a way to reduce TNFα levels in autoinflammatory diseases such as Crohn's disease and rheumatoid arthritis (Gantke *et al*, 2012). TNFα neutralisation is already used as a treatment for these conditions. Our results illustrate, however, that inhibition of TPL2 does not only lead to reduced TNFα production; levels of other cytokines such as IL-12 and IFNβ may be

increased in the absence of TPL2. Inhibition of TPL2 may therefore have harmful side-effects; increased IL-12 may lead to the development of a Th1 response and host damage through the secretion of IFNγ by T cells (although this could be beneficial in the context of bacterial infection). However, increased type I IFN could result in susceptibility to bacterial infections such as *Listeria monocytogenes* and *Mtb*. Treatments that target TPL2 should therefore be used with caution.

5.4. Figures

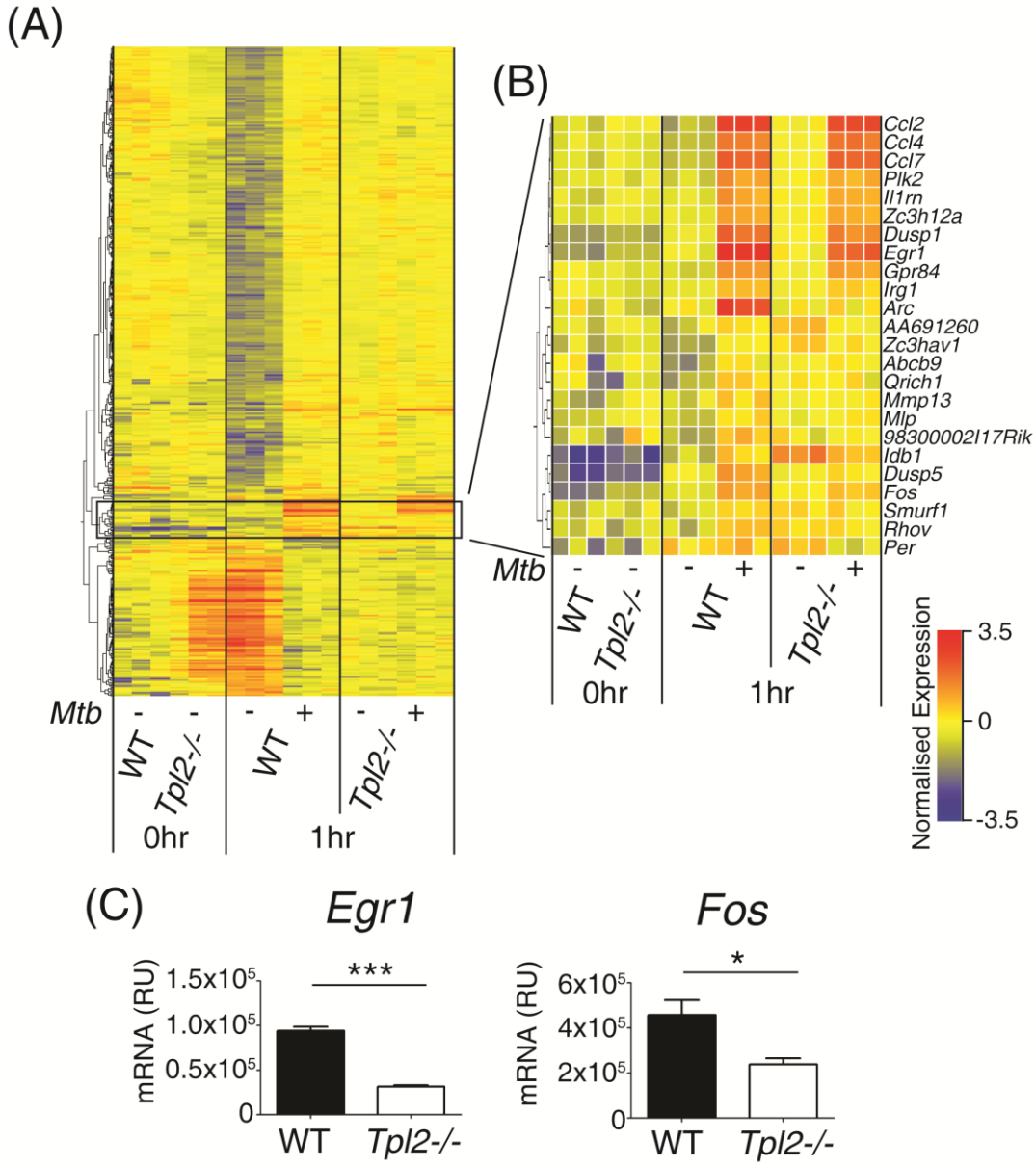

Figure 5.1. The TPL2-ERK pathway regulates early transcription in *Mtb*-infected macrophages. (A) WT and *Tpl2*$^{-/-}$ macrophages were infected with *Mtb* and at 1hr post-infection RNA was harvested and microarray analysis was carried out. Data was normalised as described in the Materials and Methods. Differentially regulated transcripts were obtained by taking those that were at least 2-fold up- or downregulated in infected samples vs. controls in either strain, and those that were significantly different by two-way ANOVA $p<0.05$ with Benjamini Hochberg FDR multi-test correction. This left 422 genes which were subjected to hierarchical clustering using Pearson centred distance metric and complete linkage (B) A cluster of 24 transcripts was found to be lower in *Tpl2*$^{-/-}$ macrophages relative to WT control. (C) The same RNA was reverse transcribed to cDNA and expression of *Egr1* and *Fos* was determined by qPCR. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; unpaired t-test.

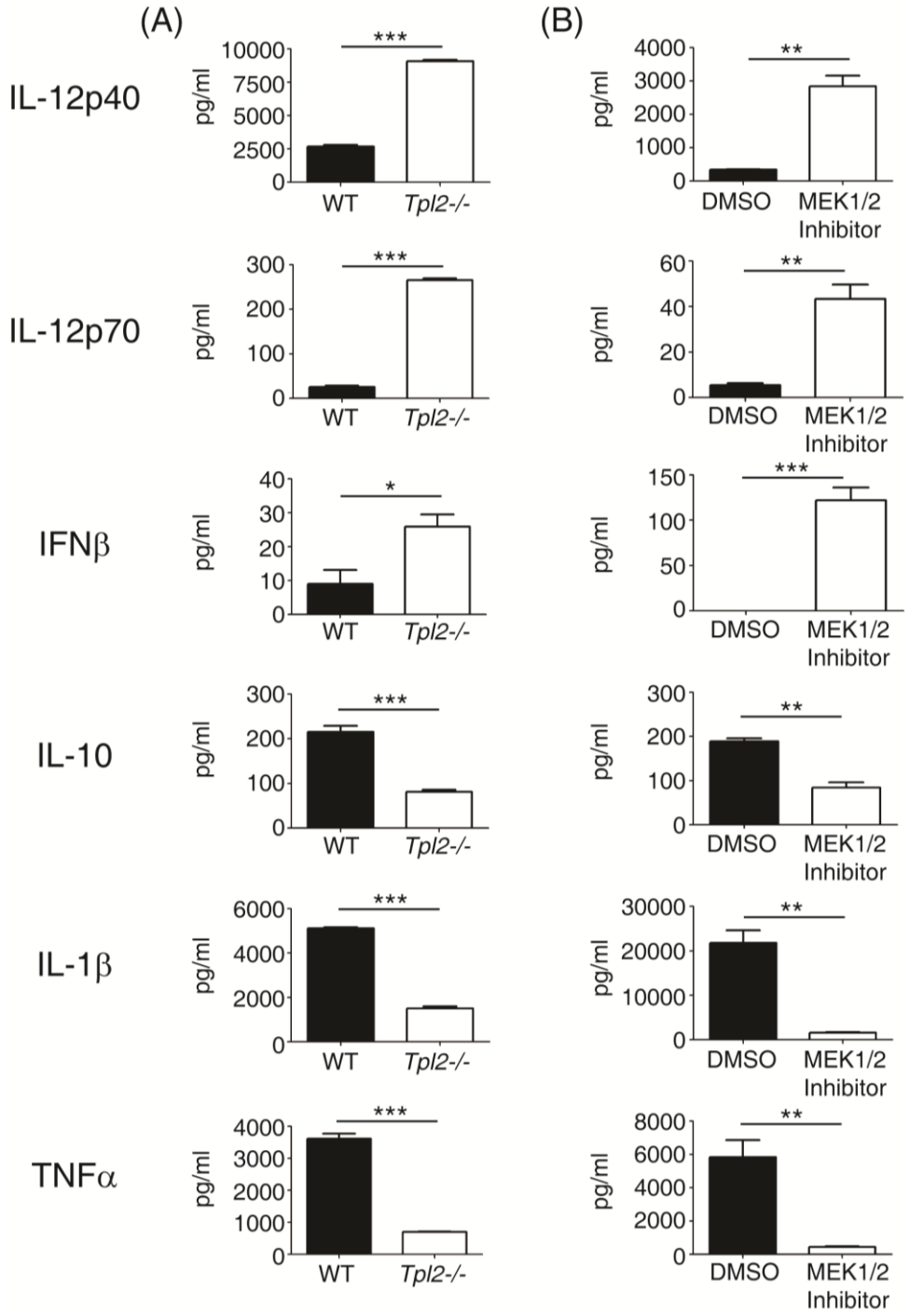

Figure 5.2. The TPL2-ERK pathway negatively regulates IL-12 and IFNβ, but positively regulates IL-10, IL-1β and TNF*α*, in *Mtb* infected macrophages. (A) WT and *Tpl2*-/- macrophages were infected with *Mtb* and cytokine levels in culture supernatant were determined at 24hr post-infection. (B) WT macrophages were pre-treated for 30mins with either the MEK1/2 inhibitor PD0325901 or DMSO vehicle control, and infected with *Mtb*. Cytokine levels in culture supernatant were determined at 24hr post-infection. Graphs show mean +/- SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; unpaired t-test. Data is representative of at least two independent experiments.

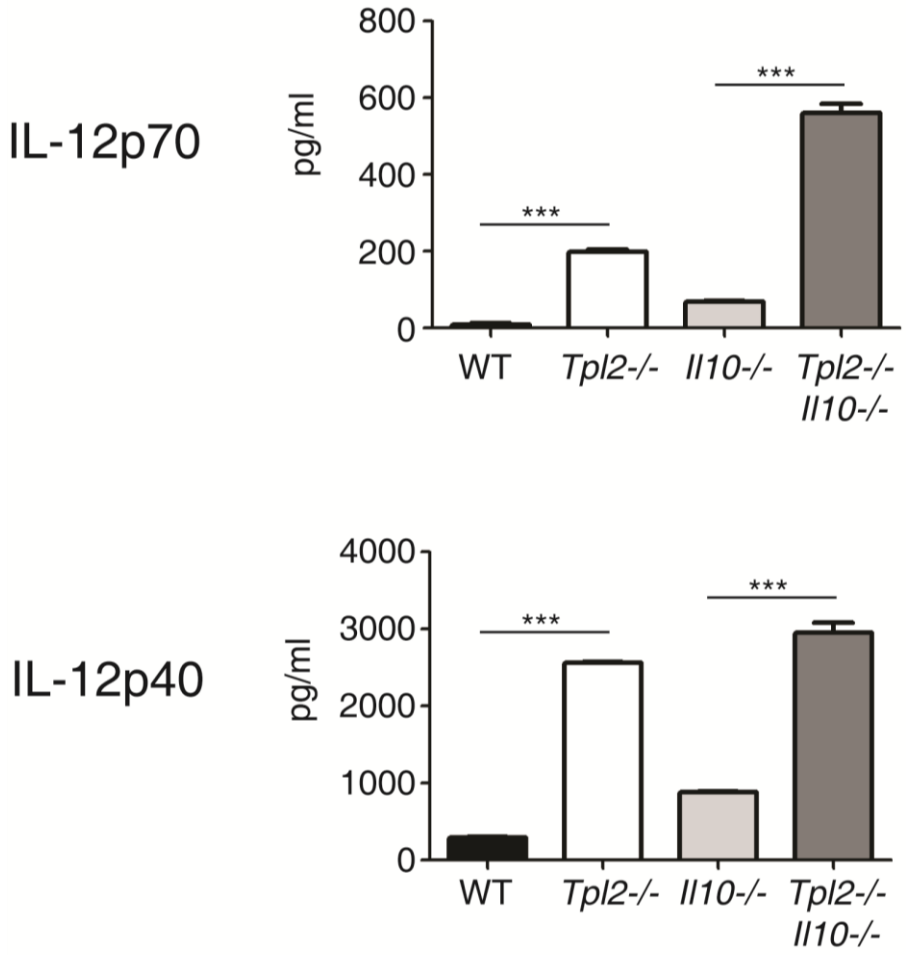

Figure 5.3. The TPL2-ERK pathway regulates IL-12 independently of IL-10. WT, *Tpl2*-/-, *Il10*-/- and *Tpl2*-/-*Il10*-/- macrophages were infected with *Mtb*. Cytokine levels in culture supernatant were determined at 24hr post-infection. Graphs show mean +/- SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; one-way ANOVA with Bonferroni post-test. Data is representative of two independent experiments.

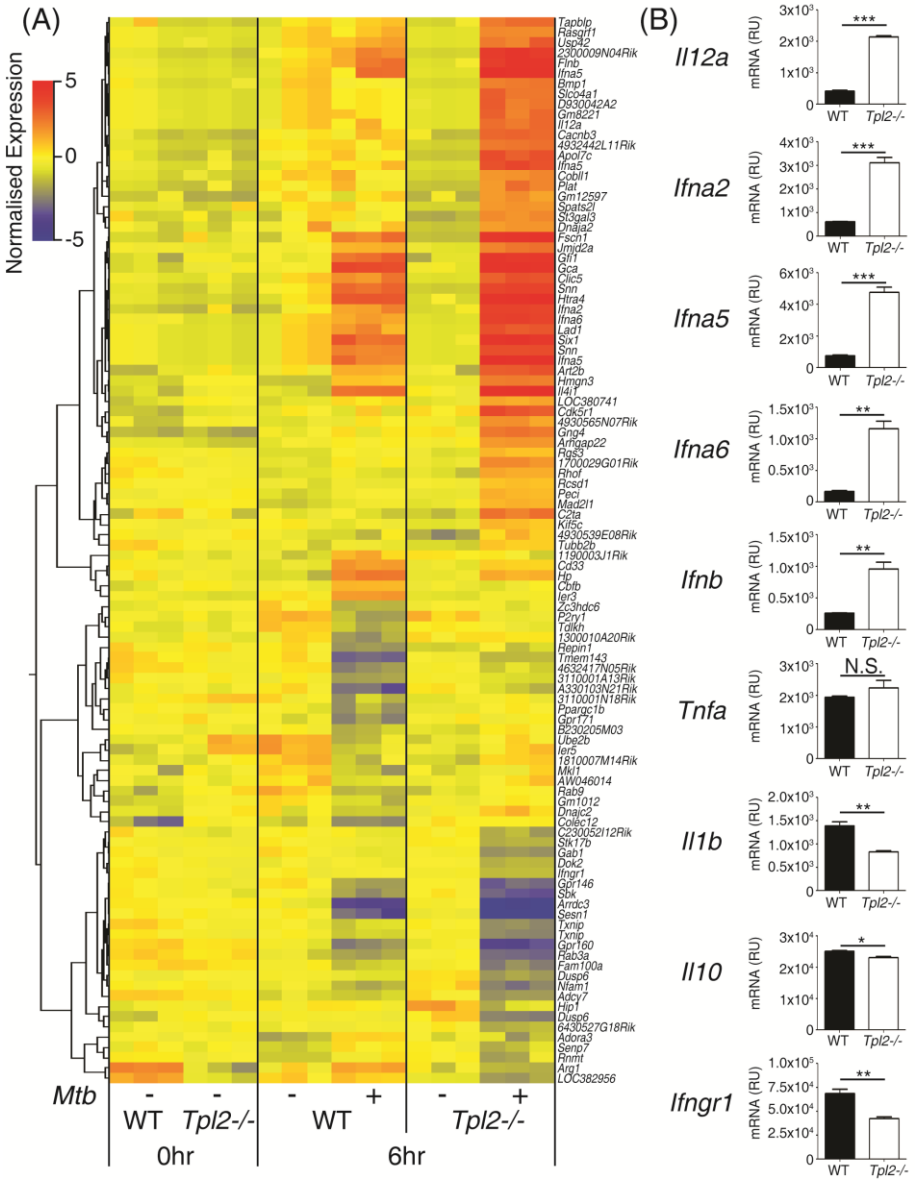

Figure 5.4. The TPL2-ERK pathway dramatically regulates transcription in *Mtb* infected macrophages. (A) WT and *Tpl2*$^{-/-}$ macrophages were infected with *Mtb* and at 0hr and 6hr post-infection RNA was harvested and microarray analysis was carried out. Data was normalised as described in the Materials and Methods and undetectable transcripts were removed. Differentially regulated transcripts were obtained by taking those that were at least 2-fold up- or downregulated in infected samples vs. controls at any time-point, and those that were significantly different by two-way ANOVA $p<0.05$ with Benjamini Hochberg FDR multiple testing correction. A further 2-fold filter between WT and *Tpl2*$^{-/-}$ infected samples at 6hr was also applied. This left 104 transcripts, which were subjected to hierarchical clustering using Pearson centred distance metric and complete linkage. Normalised expression was visualised using a heat map. (B) The same RNA was also reverse transcribed to cDNA and expression of indicated transcripts was analysed by qPCR. Graphs show mean +/- SEM (n=3). *, $p<0.05$; **, $p<0.01$, ***; $p<0.001$; N.S. not significant; unpaired t-test.

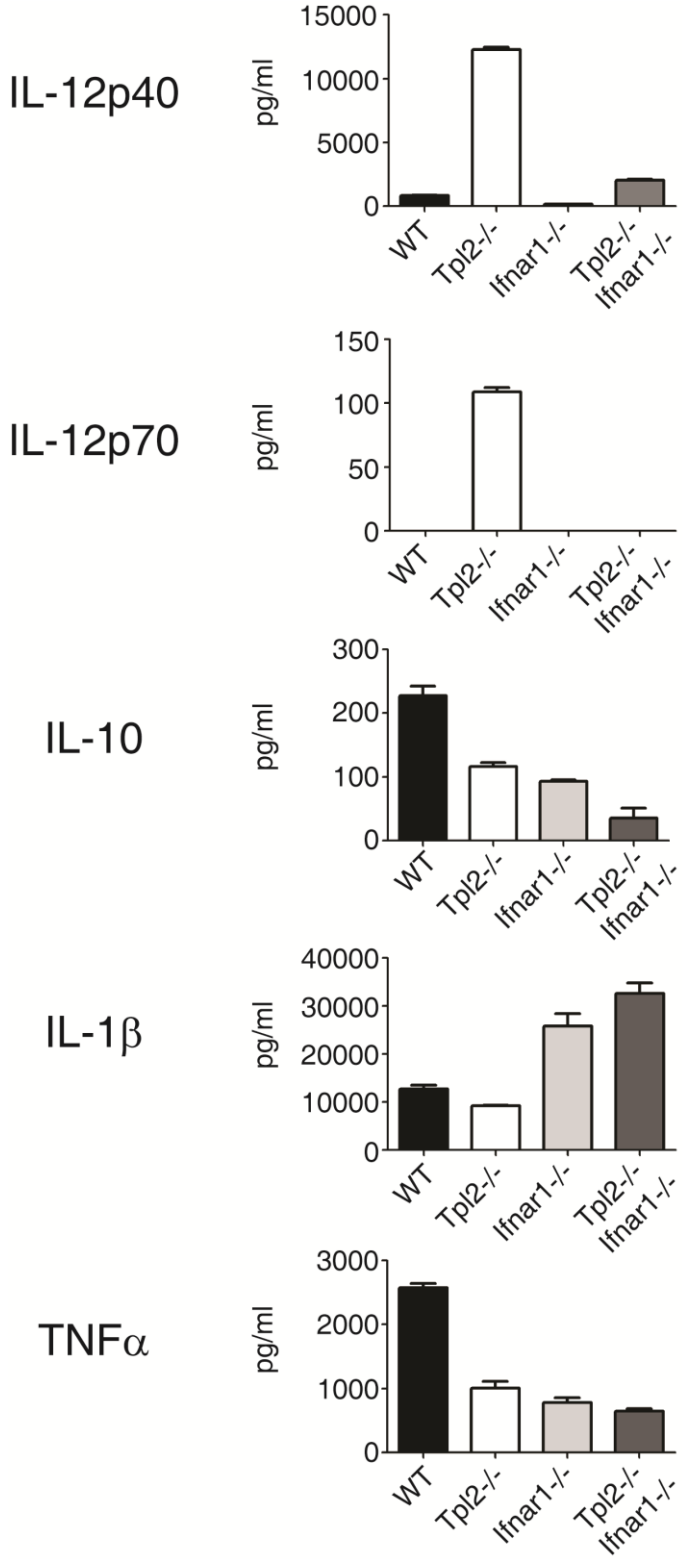

Figure 5.5. The TPL2-ERK and type I IFN pathways jointly regulate cytokine production from *Mtb* infected macrophages. WT, *Tpl2*-/-, *Ifnar1*-/- and *Tpl2*-/- *Ifnar1*-/- macrophages were infected with *Mtb*. Cytokine levels in culture supernatant were determined at 24hr post-infection. Graphs show mean +/- SEM (n=3). Data is representative of three independent experiments.

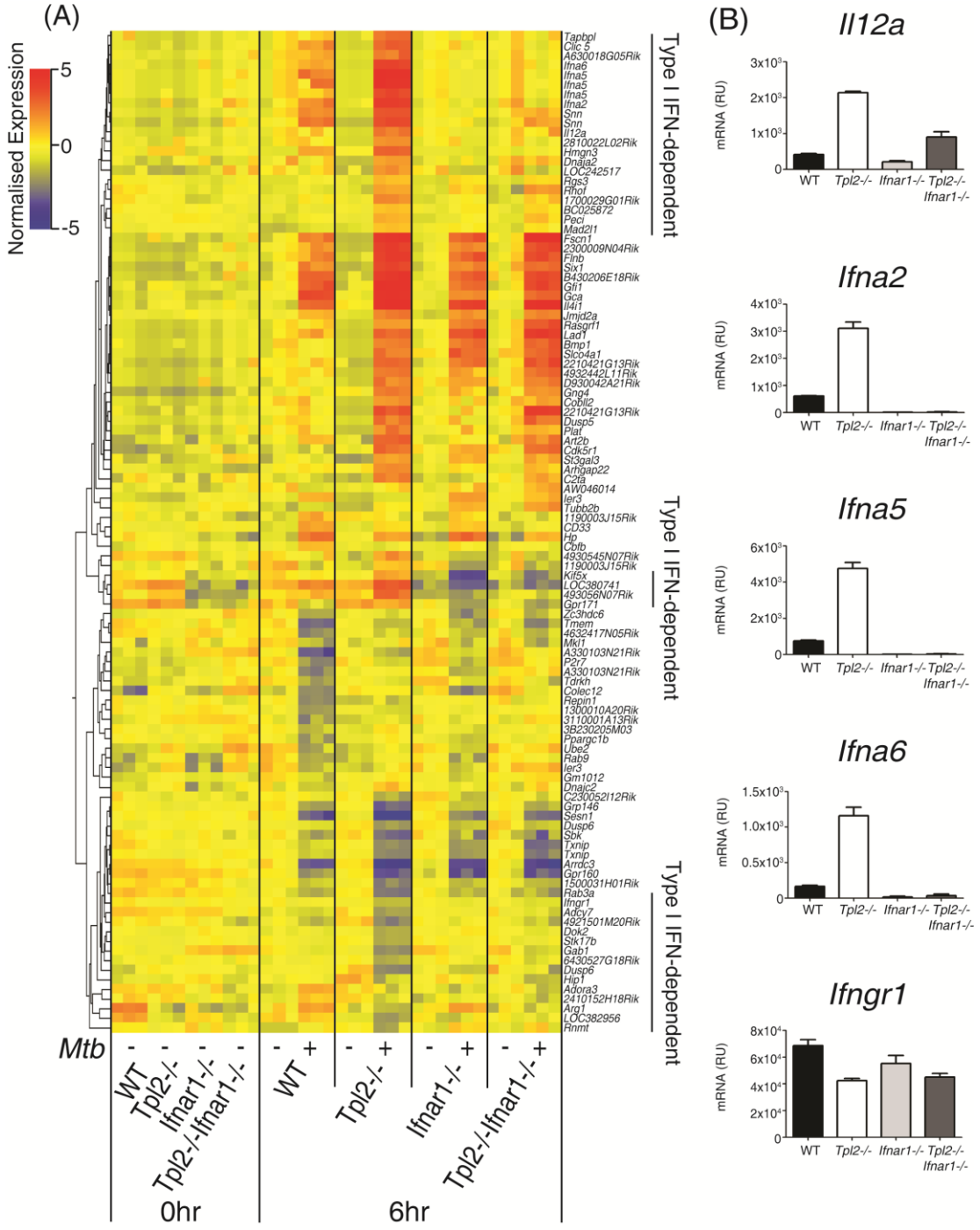

Figure 5.6. Transcriptional changes in *Tpl2*-/- macrophages are partially dependent upon type I IFN signalling. (A) WT, *Tpl2*-/-, *Ifnar1*-/- and *Tpl2*-/-*Ifnar1*-/- macrophages were infected with *Mtb* and at 0hr and 6hr post-infection RNA was harvested and microarray analysis was carried out. Data was normalised as described in the Materials and Methods and expression of the 104 transcripts in Figure 5.4 was determined. This list was subjected to hierarchical clustering using Pearson centred distance metric and average linkage. Normalised expression was visualised using a heat map. (B) The same RNA was reverse transcribed to cDNA and analysis of gene expression was carried out by qPCR. Graphs show mean +/- SEM (n=3)

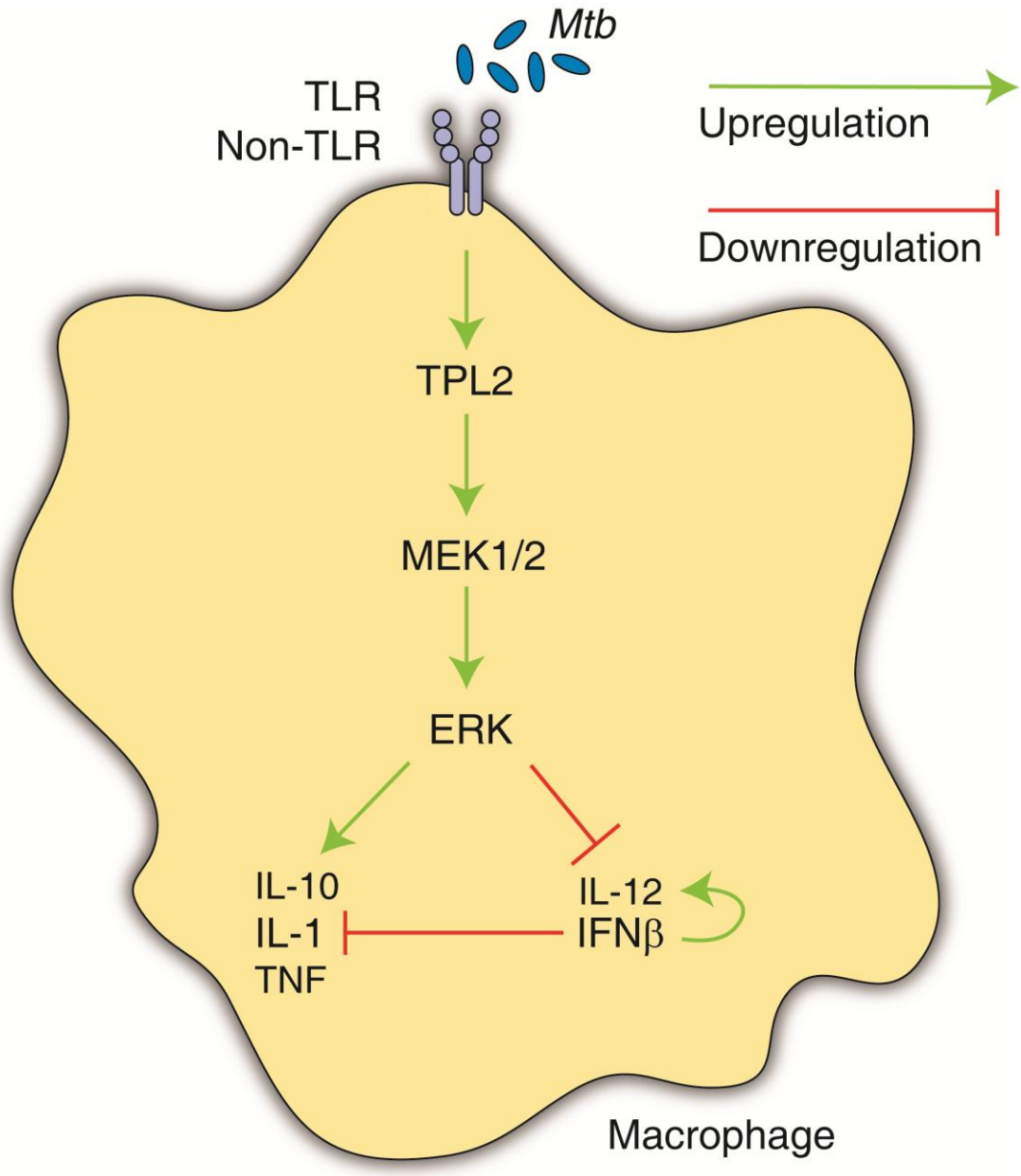

Figure 5.7. A summary of the effects of TPL2-ERK signalling on cytokine production by *Mtb*-infected macrophages

Chapter 6. Type I IFN regulates the macrophage response to IFNγ

6.1. Background

Type I IFN and IFNγ have distinct activities and affect the immune response to *Mtb* in very different ways. IFNγ is critical in protecting the host (Flynn and Chan, 2001) and mice deficient in IFNγ are unable to control infection with *Mtb* and die rapidly (Flynn *et al*, 1995; Cooper *et al*, 1995). In addition, humans with mutations in the IFNγ receptor are unable to mount a sufficient immune response to normally harmless mycobacteria, a syndrome known as Mendelian susceptibility to mycobacterial disease (MSMD) (Casanova *et al*, 2012). In contrast, type I IFN reduces host resistance to *Mtb*. Several studies found that mice deficient in the type I IFN receptor were better able to control *Mtb* infection, with reduced bacterial loads in the lung and spleen (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007). In addition, increased levels of type I IFN either in response to a hypervirulent isolate of *Mtb* (Manca *et al*, 2001) or Poly-ICLC (Antonelli *et al*, 2009) leads to increased susceptibility to *Mtb* infection in mice.

Type I IFN and IFNγ signal through separate receptors, but induce overlapping signalling pathways and overlapping sets of genes (Stark *et al*, 1998; Der *et al*, 1998). IFNγ signals through a receptor consisting of IFNGR1 and IFNGR2, whereas all type I IFNs signal through a single receptor made up of IFNAR1 and IFNAR2 (Platanias, 2005). Following binding, downstream signalling occurs through the Jak/STAT pathway (Stark *et al*, 1998). Both the type I IFN and IFNγ receptors are constitutively associated with Jak kinases; Tyk2 and Jak1 with the type I IFN receptor, and Jak1 and Jak2 with the IFNγ receptor (Stark *et al*, 1998). Binding of IFNs to their receptors causes the Jak kinases to tyrosine phosphorylate the receptors, creating docking sites for STAT proteins. Docking of STAT1 or STAT2 at the receptors results in their phosphorylation and activation, and the STAT

proteins then dimerise and translocate to the nucleus where they bind to regulatory sequences in the promoters of genes to activate transcription (Stark *et al*, 1998).

Type I IFN signalling results in both STAT1 and STAT2 phosphorylation, and these proteins form heterodimers and associate with IRF9 to form the ISGF3 complex (Platanias *et al*, 2005). ISGF3 binds to interferon stimulated response elements (ISREs) in the promoters of type I IFN inducible genes, and activates their transcription (Platanias *et al*, 2005). In contrast, IFNγ signalling only activates and phosphorylates STAT1, which forms homodimers and binds to IFNγ-activated sites (GAS) in gene promoters (Platanias *et al*, 2005). STAT1 is therefore required for the response to both type I IFN and IFNγ.

Importantly, type I IFN and IFNγ signalling can cross-regulate each other. In particular, it has been shown that low levels of constitutive type I IFN are required for an optimal response to IFNγ (Takaoka *et al*, 2000; Gough *et al*, 2011). In these studies, murine endothelial fibroblasts (MEFs) from *Ifnar1*$^{-/-}$ mice had a diminished antiviral response following addition of IFNγ (Takaoka *et al*, 2000; Gough *et al*, 2010). Takaoka *et al* (2000) suggested that this was due to an association between IFNAR1 and IFNGR2, leading to a docking site for the recruitment and activation of STAT1. This priming effect was lost in *Ifnar1*$^{-/-}$ MEFs (Takaoka *et al*, 2000). More recently, Gough *et al* (2011) suggested a mechanism whereby low constitutive type I IFN production is responsible for maintaining basal levels of STAT1, thus maintaining a pool of STAT1 which could be activated by IFNγ.

However, type I IFN was also recently shown to inhibit the response of macrophages to IFNγ. Rayamajhi *et al* (2010) found that macrophages infected with *Listeria monocytogenes* lost their responsiveness to IFNγ, with reduced STAT1 phosphorylation and reduced upregulation of MHC class II molecules on the cell

surface. This loss of responsiveness was shown to be due to type I IFN production by infected macrophages, which downregulated the IFNγ receptor subunit IFNGR1 (Rayamajhi *et al* 2010). Type I IFN has therefore been shown to both promote and inhibit IFNγ signalling.

IFNγ signalling on macrophages plays a vital role during *Mtb* infection, activating macrophages to kill intracellular *Mtb* through the induction of antimicrobial effectors such as iNOS and LRG-47 (Pieters, 2008). Type I IFN-mediated loss of macrophage responsiveness to IFNγ could therefore prevent *Mtb* killing *in vivo*. This may explain in part the increased resistance observed in *Ifnar1*$^{-/-}$ mice (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007). In addition, type I IFN may impact IFNγ signalling during human TB, as Berry *et al* (2010) showed that genes involved in both pathways are upregulated in neutrophils and monocytes in active TB patients. We therefore set out to determine how type I IFN may affect macrophage responsiveness to IFNγ in *Mtb*-infected macrophages.

6.2. Results

6.2.1. Endogenous type I IFN inhibits the induction of IL-12 by IFNγ

An important function of IFNγ is to regulate the production of cytokines in innate immune cells. IFNγ stimulates increased production of IL-12 (Ma *et al*, 2005) and TNFα from macrophages and DCs, but inhibits production of the immunosuppressive cytokine IL-10 (Hu *et al*, 2006). To determine if endogenous type I IFN could interfere with these properties of IFNγ in the context of *Mtb* infection, WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* in the presence or absence of IFNγ and the production of cytokines at 24hr post-infection was determined by ELISA.

Addition of IFNγ potently enhanced the production of IL-12p40 from *Mtb*-infected WT macrophages (Figure 6.1). However, this induction of IL-12p40 by IFNγ was significantly enhanced in the absence of endogenous type I IFN, with around 3-fold more IL-12p40 produced in *Ifnar1*$^{-/-}$ macrophages treated with IFNγ compared to WT (Figure 6.1). In addition, whereas no IL-12p70 was induced in *Mtb*-infected WT macrophages treated with IFNγ, IL-12p70 was detectable in *Ifnar1*$^{-/-}$ macrophages (Figure 6.1). This demonstrates that endogenous type I IFN can inhibit the ability of IFNγ to stimulate the production of both IL-12p40 and IL-12p70 from *Mtb*-infected macrophages.

IFNγ also stimulated enhanced TNFα production from *Mtb*-infected macrophages (Figure 6.1). However, this was unaffected by the presence of type I IFN, as a similar 3-fold increased in TNFα was observed in both WT and *Ifnar1*$^{-/-}$ macrophages (Figure 6.1). IFNγ in the absence of *Mtb* infection did not induce detectable levels of IL-12p40 or IL-12p70, but did induce a small upregulation of TNFα (data not shown).

In contrast to IL-12 and TNFα, IFNγ addition to *Mtb*-infected macrophages inhibited IL-10 production (Figure 6.1) as previously reported (Hu *et al*, 2006). Unexpectedly, given its generally pro-inflammatory properties, IFNγ also potently downregulated IL-1β production from *Mtb*-infected macrophages (Figure 6.1). However, the ability of IFNγ to suppress IL-10 and IL-1β was unaffected by endogenous type I IFN, with similar suppression observed in WT and *Ifnar1*$^{-/-}$ macrophages (Figure 6.1). Taken together, this shows that endogenous type I IFN specifically inhibits the induction of IL-12p40 and IL-12p70 by IFNγ in *Mtb*-infected macrophages. As IL-12 is crucial in protecting the host against *Mtb* infection due to its ability to stimulate the Th1 response (Cooper *et al*, 1997), this may have detrimental consequences for the immune response to *Mtb*.

6.2.2. Exogenous type I IFN blocks the regulation of IL-12p40 and IL-10 by IFNγ

In vivo, type I IFN can be made by many cell types and may act upon infected macrophages in a paracrine manner (Theofilopoulos *et al*, 2005). Having previously shown that endogenous type I IFN could interfere with the ability of IFNγ to stimulate IL-12 production, we next investigated whether addition of exogenous IFNβ had similar effects. *Mtb*-infected macrophages were treated with IFNβ alone, IFNγ alone and a combination of IFNβ and IFNγ, with IFNs added at the time of infection.

As shown in Figure 6.2, IFNγ enhanced IL-12p40 production, whereas IFNβ inhibited it, as previously shown. However, if IFNβ and IFNγ were added together, there was minimal upregulation of IL-12p40 (Figure 6.2). Exogenous IFNβ can therefore block the ability of IFNγ to induce IL-12p40 production, similar to the

activities of endogenous type I IFN. In the absence of IFNβ, IFNγ suppressed IL-10 production by around 2-fold and addition of IFNβ enhanced IL-10 production (Figure 6.2). In contrast, addition of IFNβ to *Mtb*-infected macrophages increased IL-10 production (Figure 6.2) IFNβ also blocked the downregulation of IL-10 by IFNγ; addition of IFNγ in combination with IFNβ had no effect on IL-10 production relative to IFNβ alone (Figure 6.2).

In contrast, the ability of IFNγ to suppress IL-1β production was not affected by IFNβ. Both IFNβ and IFNγ suppressed IL-1β production, with IFNγ having the more potent effect (Figure 6.2). However, addition of both IFNs together caused an additive decrease in IL-1β production (Figure 6.2) showing that the ability of IFNγ to suppress IL-1β is not blocked by IFNβ. In addition, the ability of IFNγ to upregulate TNFα production from *Mtb*-infected macrophages was unaffected by IFNβ (Figure 6.2). Addition of IFNγ led to a moderate increase of TNFα compared to *Mtb* alone, and a similar increase was observed if IFNβ was added concomitantly (Figure 6.2). Together, these results show that exogenous IFNβ specifically interferes with the ability of IFNγ to enhance IL-12 and inhibit IL-10 from *Mtb*-infected macrophages.

6.2.3. Endogenous type I IFN can inhibit IFNγ-induced killing of *Mtb*

The key function of IFNγ in the context of *Mtb* infection is to activate macrophages to kill intracellular bacteria (Flynn and Chan, 2001). Given that endogenous or exogenous type I IFN could inhibit the ability of IFNγ to modulate cytokine production from macrophages, it was of interest to determine if it could also block IFNγ-mediated bacterial killing. To address this, WT and *Ifnar1*$^{-/-}$

macrophages were infected with *Mtb* in the presence or absence of IFNγ and intracellular bacterial levels were assessed at 96hr post-infection.

Addition of IFNγ led to a significant 2-fold reduction in *Mtb* at 96hr in WT macrophages (Figure 6.3). However, in *Ifnar1*$^{-/-}$ macrophages this effect was more profound, with around a 4-fold reduction in *Mtb* levels (Figure 6.3). This was significantly lower than *Mtb* levels in WT macrophages stimulated with IFNγ (Figure 6.3). Endogenous type I IFN can therefore inhibit killing of *Mtb* by IFNγ.

6.2.4. Type I IFN regulates IFNγ-induced transcription in *Mtb*-infected macrophages

IFNγ induces the transcription of numerous genes in order to mediate its effects (Der *et al*, 1998). Having shown above that endogenous type I IFN can inhibit the upregulation of IL-12 by IFNγ, and IFNγ-mediated killing, we next investigated the global effects of endogenous type I IFN on IFNγ-induced gene expression. This could give insights into additional functions of IFNγ that may be regulated by type I IFN, and may also suggest mechanisms for the effects observed previously. For this experiment we chose to treat WT and *Ifnar1*$^{-/-}$ macrophages with IFNγ in the absence of *Mtb*, in order to specifically address the role of basal type I IFN on IFNγ-induced gene expression, as basal type I IFN has been reported to be a major regulator of IFNγ signalling (Takaoka *et al*, 2000; Gough *et al*, 2010).

WT and *Ifnar1*$^{-/-}$ macrophages were treated with IFNγ at 5ng/ml and RNA was harvested at baseline and at 6hr post-treatment. The RNA was then purified, amplified and hybridised to Illumina Mouse WG-6 V2.0 Beadchip arrays, as described in the Materials and Methods, with triplicate biological samples for each condition. Following normalisation and removal of undetectable transcripts as

described in the Materials and Methods. Further analysis was then used to identify IFNγ-inducible transcripts that were regulated by endogenous type I IFN. First, transcripts were retained if they were up- or downregulated by IFNγ at 6hr in either WT or *Ifnar1*$^{-/-}$ macrophages, relative to untreated controls at 6hr. Second, transcripts were retained if they were statistically different between WT and *Ifnar1*$^{-/-}$ macrophages by two-way ANOVA with Benjamini Hochberg FDR multiple testing correction. This identified 396 transcripts that were differentially regulated between WT and *Ifnar1*$^{-/-}$ macrophages. These 396 genes were clustered hierarchically using Pearson centred distance metric and complete linkage (Figure 6.4).

As shown in Figure 6.4, type I IFN has diverse effects on IFNγ-induced transcription. A prominent cluster of transcripts was induced to higher levels by IFNγ in *Ifnar1*$^{-/-}$ macrophages compared to WT macrophages, in keeping with an inhibition of IFNγ function describe previously (Figure 6.4). However, IFNγ induction of other transcripts was dependent upon type I IFN, with reduced induction observed in *Ifnar1*$^{-/-}$ macrophages (Figure 6.4). In addition, a number of IFNγ inducible transcripts were regulated by basal type I IFN (Figure 6.4).

To separate these clusters for further analysis, *k*-means clustering was applied to separate the 396 transcripts into six clusters. Expression profiles and heat maps of these six clusters are shown in Figure 6.5, with relevant genes shown from each cluster. Complete lists of the transcripts found in each cluster are presented in section 6.5 at the end of this Chapter.

The transcripts in cluster 0 and cluster 1 were upregulated by IFNγ in WT macrophages, but this was dependent upon endogenous type I IFN, as reduced upregulation was seen in *Ifnar1*$^{-/-}$ macrophages (Figure 6.5). Transcripts in cluster 1 were completely dependent upon type I IFN for their expression, whereas transcripts

in cluster 0 showed partial dependence (Figure 6.5). Cluster 0 included the apoptosis related genes *Fas* and *Casp8* (Caspase 8), suggesting that IFNγ may induce apoptosis in macrophages, in a type I IFN dependent manner. This may be a mechanism to reduce the viability of *Mtb* and increase cross-presentation (Behar *et al*, 2011). Cluster 0 also included the IL-1 receptor antagonist *Il1rn*. Both type I IFN and IFNγ suppress IL-1β production (Figure 6.1 and Chapter 4) but this data suggests that IFNs may also neutralise IL-1 through *Il1rn* production. Cluster 1, a small cluster of 24 transcripts, included the antiviral gene *Isg20*, in line with reported type I IFN-dependent antiviral activity of IFNγ (Takaoka *et al*, 2000; Gough *et al*, 2010b).

Transcripts in cluster 4 and cluster 5 were distinguished by being heavily dependent on type I IFN for expression at baseline, with dramatically lower expression in *Ifnar1*$^{-/-}$ medium controls at 0hr and 6hr compared to WT (Figure 6.5). However, these transcripts were strongly induced by IFNγ in WT macrophages at 6hr post-treatment. This shows that transcripts induced by type I IFN and IFNγ overlap, as previously reported (Der *et al*, 1998). Cluster 4 includes transcripts which, although being dramatically lower at baseline, are strongly induced in *Ifnar1*$^{-/-}$ macrophages by IFNγ, to levels comparable to the expression observed in WT macrophages treated with IFNγ, showing that IFNγ can effectively compensate for the lack of endogenous type I IFN for these genes. Genes in this cluster include the GTPases *Gbp2*, *Gbp4*, *Gbp5*, *Gbp6*, the transcription factor *Stat1*, the components of the TAP transporter *Tap1* and *Tap2*, the chemokine *Cxcl10* and *Irgm1* (LRG-47). It is notable that genes which are considered to be primarily IFNγ-inducible, such as the *Gbps* (MacMicking, 2004), are strongly regulated by endogenous type I IFN.

Cluster 5 contains transcripts which are also heavily regulated at baseline by type I IFN, but in contrast to genes in Cluster 2, addition of IFNγ only partially restores transcription, and levels remain much lower than those seen in WT macrophages (Figure 6.5). Genes in this cluster included *Oas1g*, *Oas2*, *Oas2l*, *Prkr*; genes with prominent antiviral functions (Sadler and Williams, 2008). The fact that IFNγ cannot fully induce these antiviral genes in *Ifnar1*$^{-/-}$ macrophages may result in a loss of IFNγ-mediated antiviral activity, as previously reported (Takaoka *et al*, 2000; Gough *et al*, 2010b). Cluster 5 also contained the transcription factors *Stat2* and *Irf7*.

Transcripts in cluster 3 were induced to higher levels by IFNγ in *Ifnar1*$^{-/-}$ macrophages compared to WT, showing endogenous type I IFN can inhibit responsiveness to IFNγ, but only in the case of specific genes (Figure 6.5) in keeping with previous findings for cytokine regulation by IFNγ (Figure 6.1). Genes in this cluster include many MHC class II molecules such as *H2*-Aa, *H2-Ab1*, *H2-DMa*, *H2-DMb1*, *H2-DMb2* and *H2-Eb1*. Cluster 4 also contains *li* (CD74) the invariant chain of MHC class II complex (Vyas *et al*, 2008). This suggests that type I IFN may be able to suppress the induction of antigen presentation by IFNγ via the MHC class II pathway. This could be important, as antigen presentation via MHC class II is vital for the induction of the protective $CD4^+$ T cell response (Cooper, 2009).

Finally, Cluster 2 contains genes that are downregulated to a greater extent in *Ifnar1*$^{-/-}$ macrophages compared to WT (Figure 6.5). This cluster includes *Cd28*, *Tlr7*, *Tlr8*, *Dusp1*, *Dusp4* and *Dusp6*.

6.2.5. Reduced STAT1 phosphorylation by IFNγ in the absence of endogenous type I IFN

The predominant transcription factor activated in response to IFNγ signalling is STAT1, which forms homodimers and travels to the nucleus where it mediates the induction of IFNγ-inducible genes (Stark *et al*, 1998). Given that endogenous type I IFN dramatically regulates IFNγ induced transcription, and that type I IFN also signals through STAT1, the effect of type I IFN on this key signalling pathway was investigated by Western blotting. WT and *Ifnar1*$^{-/-}$ macrophages were stimulated with IFNγ and lysates were collected at 0hr, 7.5mins, 15mins, 30mins and 1hr post-stimulation. These were probed with antibodies against total STAT1, STAT1 phosphorylated on tyrosine residue 701, the major phosphorylation site for STAT1 activation (Platanias, 2005), or actin as a loading control.

Levels of total STAT1 were dramatically reduced in *Ifnar1*$^{-/-}$ macrophages (Figure 6.6), which is in keeping with the fact that STAT1 mRNA levels are also much lower in *Ifnar1*$^{-/-}$ macrophages, as measured by microarray (Figure 6.5). This is in keeping with a previous report by Gough *et al* (2010), showing loss of basal STAT1 in *Ifnar1*$^{-/-}$ macrophages MEFs. As expected, addition of IFNγ to WT macrophages led to rapid phosphorylation of STAT1 in WT macrophages (Figure 6.6). However, IFNγ induced STAT1 phosphorylation in *Ifnar1*$^{-/-}$ macrophages was greatly reduced, possibly reflecting the lower levels of total STAT1 in *Ifnar1*$^{-/-}$ macrophages (Figure 6.6). This may explain why certain genes are induced at lower levels by IFNγ in *Ifnar1*$^{-/-}$ macrophages.

6.2.6. Type I IFN can regulate mRNA levels of the IFNγ receptor

It has been previously reported that in macrophages infected with *Listeria monocytogenes*, type I IFN could inhibit the expression of the IFNGR1 subunit of the IFNγ receptor (Rayamajhi *et al*, 2010). This may explain the increased induction of some IFNγ inducible genes in *Ifnar1*$^{-/-}$ macrophages, as levels of the IFNγ receptor, and therefore IFNγ signalling, would be increased. To determine if type I IFN was capable of downregulating *Ifngr1* in our system, exogenous IFNβ was added to WT macrophages, RNA was harvested and levels of *Ifngr1* were measured by qPCR. As shown in figure 6.7A, addition of IFNβ led to a reduction in *Ifngr1* mRNA levels after 6hr of stimulation. This may partially explain how IFNβ is able to block the effects of IFNγ on cytokine regulation (Figure 6.2).

We next determined whether *Mtb* infection of macrophages led to a downregulation of *Ifngr1* mRNA, and whether this was dependent upon endogenous type I IFN, as was reported for *Listeria monocytogenes* infection of macrophages (Rayamajhi *et al*, 2010). WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* and mRNA levels of *Ifngr1* were measured by qPCR. As shown in Figure 6.7B, *Mtb* infection resulted in a downregulation of *Ifngr1* in WT macrophages at 6hr post-infection. This was independent of type I IFN, as a similar effect was seen in *Ifnar1*$^{-/-}$ macrophages. This may represent an important virulence strategy by *Mtb* to subvert IFNγ mediated killing in macrophages. However, unlike for *Listeria monocytogenes* infection, this was independent of type I IFN.

6.3. Discussion

Both type I IFN and IFNγ are produced during *Mtb* infection, but these two cytokines have opposite effects on host resistance to *Mtb*. Whereas IFNγ is critical for controlling *Mtb* infection (Flynn *et al*, 1995; Cooper *et al*, 1995), type I IFN reduces host resistance (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007). Previous reports have shown that type I IFN and IFNγ can cross-regulate each other, with both positive and negative effects of type I IFN on IFNγ signalling reported (Takaoka *et al*, 2000; Rayamajhi *et al* 2010; Gough *et al*, 2010b). Here we show that type I IFN could inhibit IFNγ-mediated cytokine regulation, and IFNγ-mediated *Mtb* killing, in macrophages. However, transcriptional analysis revealed additional complexity, with type I IFN both positively and negatively regulating IFNγ-induced gene expression. Given the vital role of IFNγ in protecting against *Mtb*, this may have important consequences for the *in vivo* immune response to *Mtb*.

6.3.1. Type I IFN inhibits key functions of IFNγ

Rayamajhi *et al* (2010) reported that type I IFN inhibited macrophage responsiveness to IFNγ in the context of *Listeria monocytogenes* infection. This study found that the ability of IFNγ to stimulate MHC class II presentation on the macrophage surface was enhanced in the absence of type I IFN (Rayamajhi *et al* 2010). This is in keeping with the results of our microarray analysis, showing that a number of MHC class II molecules, and the invariant chain *li* (CD74), were induced to a greater extent by IFNγ in *Ifnar1*$^{-/-}$ macrophages at the mRNA level. However, we also found that two further key functions of IFNγ were inhibited by type I IFN. First, type I IFN inhibited the ability of IFNγ to upregulate IL-12, and downregulate IL-10 from *Mtb*-infected macrophages. Second, type I IFN inhibited the ability of

IFNγ to stimulate *Mtb*-killing in macrophages. The inhibition of these activities of IFNγ *in vivo* by type I IFN may have profound effects on host resistance, and may go some way to explaining why mice deficient in type I IFN signalling mount a more effective immune response to *Mtb* (Manca *et al*, 2005; Ordway *et al*, 2007; Stanley *et al*, 2007).

IFNγ is a potent activator of macrophages, and induces several pathways that result in the killing of *Mtb*, including induction of the enzyme iNOS, which generates antimicrobial free radicals such as NO_2, the stimulation of autophagy, which leads to the destruction of *Mtb* in the phagosome, and the induction of several members of the GTPase family. T cell derived IFNγ is therefore thought to be the main mechanism by which *Mtb* is eliminated from the body (Flynn and Chan, 2001). However, *Mtb* is capable of surviving in the face of this robust Th1 mediated response, establishing a latent infection that can reactivate many years later. *Mtb* is therefore likely to have evolved a number of strategies to avoid and subvert this IFNγ-mediated Th1 response, in order to persist. Our results suggest that the induction of type I IFN in macrophages may be one such virulence strategy to blunt IFNγ killing.

As discussed above, IFNγ activates several different killing mechanisms in *Mtb*-infected macrophages. Microarray analysis of WT and *Ifnar1*$^{-/-}$ macrophages found a number of IFNγ-inducible genes to be more strongly induced in *Ifnar1*$^{-/-}$ macrophages. However, this did not include *Nos2* (iNOS), *Irgm1* (LRG-47) or members of the GTPase family. This does not rule out an inhibition of these factors by type I IFN; the microarray was only carried out at one time point (6hr) and thus the kinetics of the induction of these genes by IFNγ may have been altered by type I IFN. For example, these genes may have been induced more rapidly by IFNγ in the

absence of type I IFN. Alternatively, their induction may have been maintained over a longer period. Future analyses could repeat these experiments over several time points to address this.

A further important function of IFNγ during the immune response is to stimulate cells such as macrophages and DCs to present antigens to naive T cells. IFNγ stimulated both MHC class I and class II antigen presentation. Induction of MHC class II genes occurs via the induction of the class II transactivator, a master regulator of class II gene expression (Boehm *et al*, 1997). In keeping with this, *C2ta*, encoding the class II transactivator, and many MHC class II molecules were strongly induced by IFNγ in macrophages. Strikingly, several MHC class II molecules were more strongly upregulated in *Ifnar1*$^{-/-}$ macrophages, suggesting that endogenous type I IFN may block the ability of IFNγ to induce antigen presentation. Presentation of *Mtb* antigens on MHC class II molecules by APCs is a key stage in *Mtb* infection, as it leads to the activation of the protective CD4^{+} T cell response (Cooper, 2009). Further work should confirm whether type I IFN can block antigen presentation, in particular focussing on DCs, as these are known to activate CD4^{+} T cells in the lymph nodes during *Mtb* infection (Wolf *et al*, 2007; Reiley *et al*, 2008). For example, the ability of IFNγ-stimulated WT and *Ifnar1*$^{-/-}$ DCs to induce T cell proliferation or IFNγ production *in vitro* could be assessed.

These results are reminiscent of several previous studies showing that *Mtb* could inhibit the macrophage response to IFNγ (Ting *et al*, 1999; Kincaid and Ernst, 2003; Nagabhushanam *et al*, 2003; Fortune *et al*, 2004). These studies found that *Mtb*-infected macrophages showed reduced ability to upregulate MHC class II expression following IFNγ treatment, and this was found to be partially dependent upon IL-6 production from *Mtb*-infected macrophages (Nagabhushanam *et al*, 2003)

but seemed to be independent of effects on JAK-STAT signalling (Ting *et al*, 1999; Kincaid and Ernst, 2003). In addition, the suppression of IFNγ responsiveness by *Mtb* was shown to be both MyD88-dependent and -independent (Fortune *et al*, 2004). Our results suggest that induction of type I IFN by *Mtb*-infected macrophages may represent an additional mechanism for inhibiting IFNγ responsiveness, and this may account for the observed MyD88-independent effects (Fortune *et al*, 2004), as type I IFN production in response to *Mtb* is MyD88-independent (Stanley *et al*, 2007; Pandey *et al*, 2009).

6.3.2. Mechanisms behind type I IFN and IFNγ cross-talk

An important question following the observation that type I IFN could block the activities of IFNγ was how this occurs. One possible explanation, put forward by Rayamajhi *et al* (2010), was that type I IFN downregulates expression of the IFNγ receptor, specifically the *Ifngr1* subunit. In keeping with these observations, we found that addition of IFNβ to macrophages led to a downregulation of *Ifngr1* at the mRNA level. However, IFNβ downregulation of *Ifngr1* only occurred after 6hr of treatment. This suggests that pre-treatment of macrophages with IFNβ would be required to inhibit responsiveness to IFNγ. However, we found that addition of IFNβ at the same time as IFNγ was able to block the regulation of cytokines by IFNγ. It therefore seems likely that other mechanisms may be involved.

A second likely point of cross-regulation between type I IFN and IFNγ signalling is at the level of STAT1 activation, as both type I IFN and IFNγ signal through STAT1 (Platanias, 2005). We found that endogenous type I IFN was required for maximal STAT1 activation by IFNγ. This is in keeping with a study by Gough *et al* (2010) which found that in MEFs, endogenous type I IFN was required

was optimal STAT1 phosphorylation in response to IFNγ, and also for IFNγ-induced antiviral activity. Indeed, we found that the induction of many antiviral genes by IFNγ, including *Mx1*, *Oas* family members and *Prkr* was found to be dependent upon endogenous type I IFN, which may explain the loss of antiviral activity by Gough *et al* (2010).

An important finding of this study is that endogenous type I IFN can regulate IFNγ-induced gene expression in a variety of ways in macrophages. Previous studies have shown both a positive (Gough *et al*, 2010) and negative (Rayamajhi *et al*, 2010) effect of type I IFN on IFNγ activities. Our microarray analysis found that both positive and negative effects can occur simultaneously, in a gene specific manner. IFNγ induced clusters of genes were identified that were induced to both higher levels, showing negative regulation, and lower levels, showing positive regulation, in *Ifnar1*$^{-/-}$ macrophages. This illustrates the complexity of type I IFN and IFNγ cross-talk, and suggests that several mechanisms of regulation may occur simultaneously. Regulation of both *Ifngr1* and STAT1 phosphorylation may play a part in this. In addition, this may have important consequences for the host response to *Mtb* infection *in vivo*.

6.4. Figures

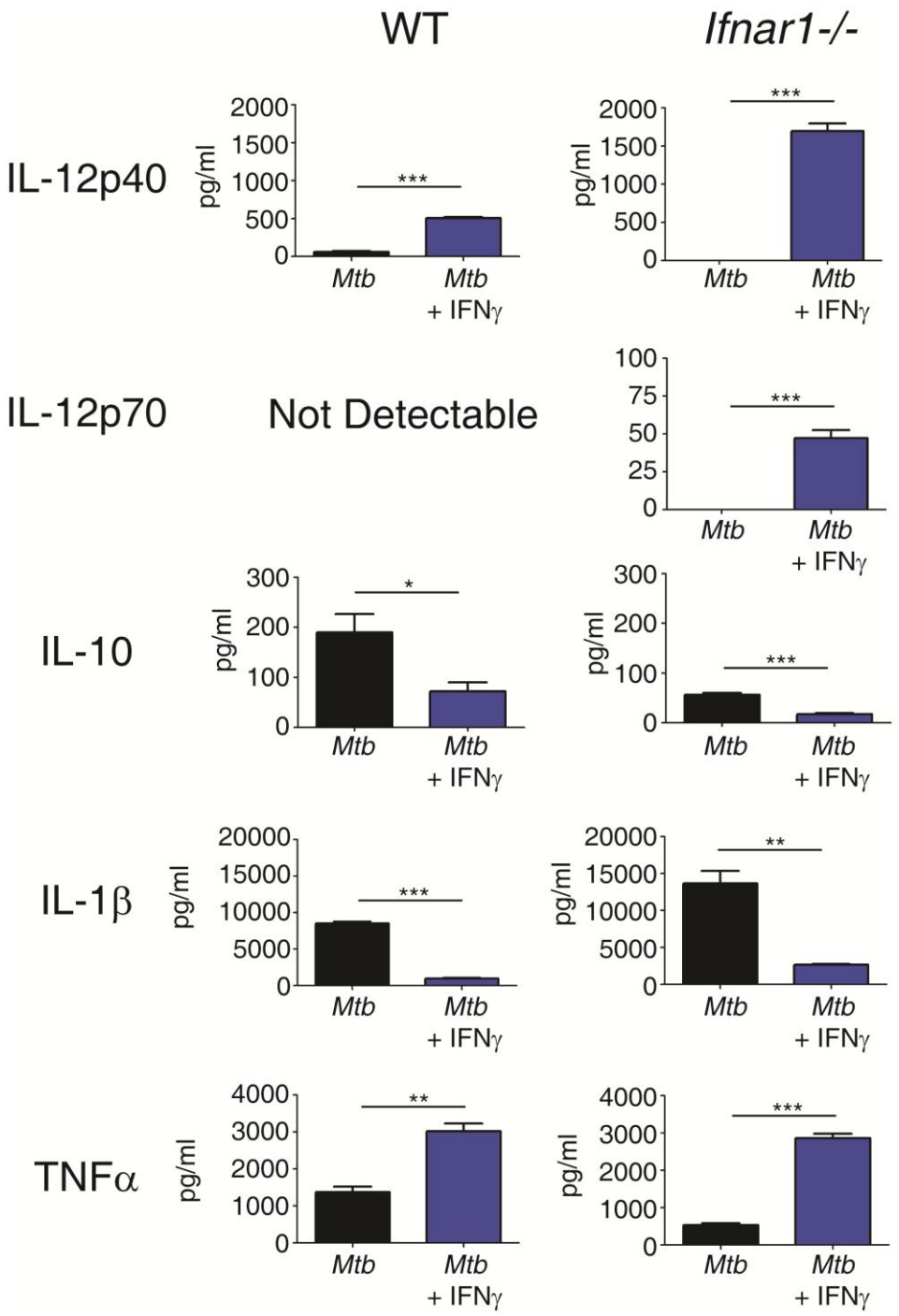

Figure 6.1. Type I IFN signalling inhibits the ability of IFNγ to upregulate IL-12 production from *Mtb*-infected macrophages. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* in the presence or absence of IFNγ, added concomitantly at 5ng/ml. Cytokine levels in culture supernatant were determined at 24hr post-infection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$; unpaired t-test. Graphs show mean +/- SEM (n=3). Data is representative of at least three independent experiments.

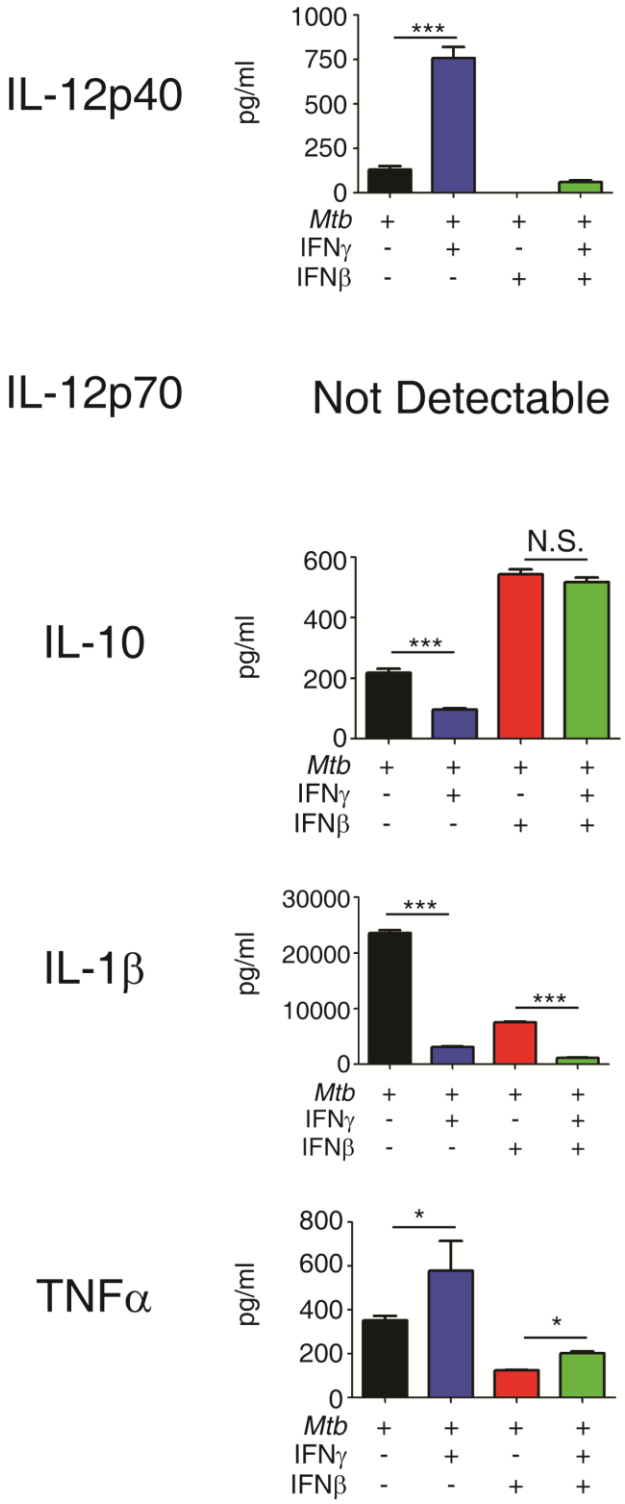

Figure 6.2. Exogenous IFNβ blocks the ability of macrophages to induce IL-12, and suppress IL-10, from *Mtb*-infected macrophages. WT macrophages were infected with *Mtb* in the presence or absence of IFNβ (2ng/ml), IFNγ (5ng/ml) or both together. Cytokine levels in culture supernatant were determined at 24hr post-infection. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, N.S. not significant; unpaired student's t-test. Graphs show mean +/- SEM (n=3). Data is representative of three independent experiments.

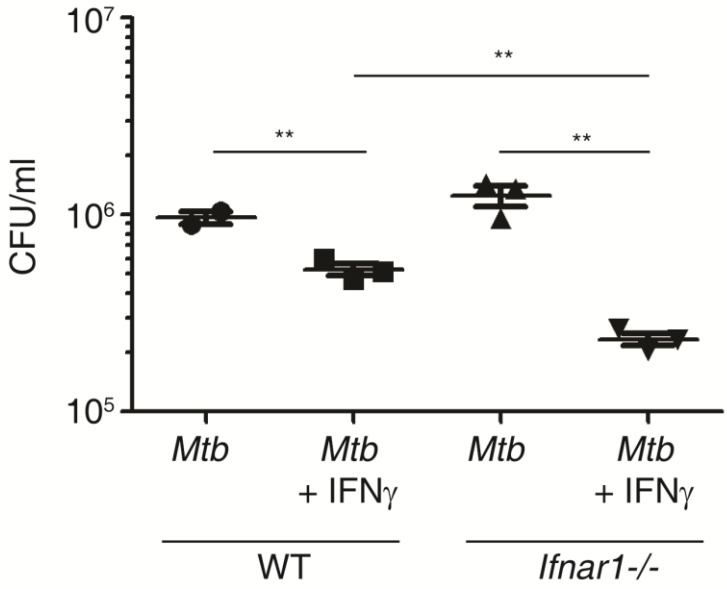

Figure 6.3. Endogenous type I IFN inhibits IFNγ-induced killing of *Mtb* in macrophages. WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb* in the presence or absence of IFNγ, added concomitantly at 5ng/ml. At 96hr post-infection cell were lysed with 0.2% saponin and bacterial counts were determined by plating onto 7H11 plates supplemented with OADC. **, $p<0.01$; unpaired t-test. Data is representative of two independent experiments.

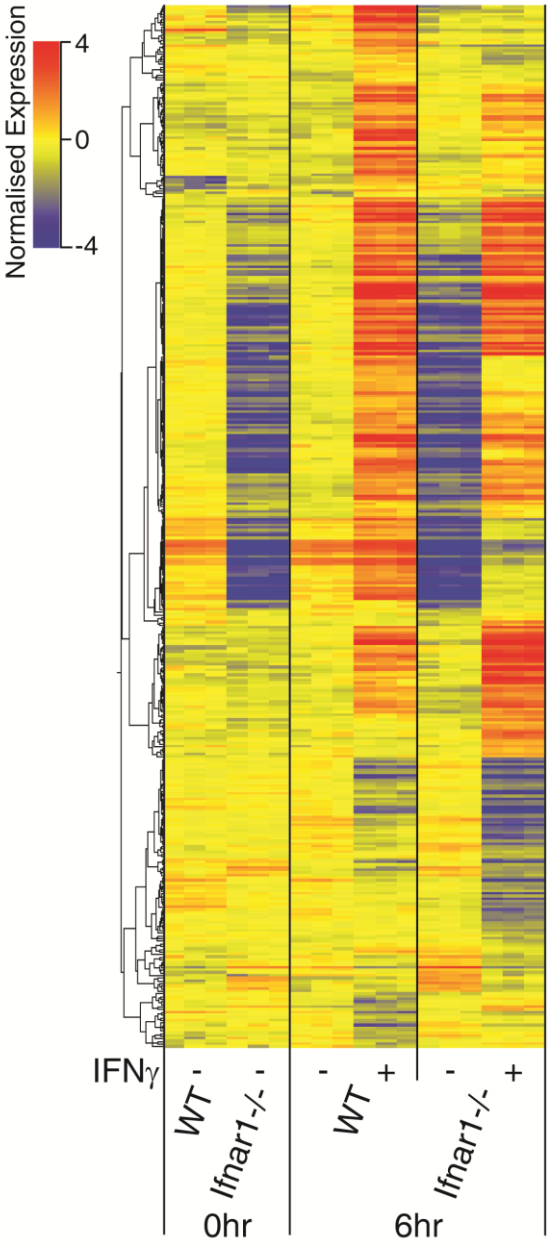

Figure 6.4. Endogenous type I IFN dramatically regulates the macrophage transcriptional response to IFNγ. (A) WT and *Ifnar1*$^{-/-}$ macrophages were stimulated with IFNγ at 5ng/ml and at 0hr and 6hr post-infection RNA was harvested and microarray analysis was carried out. Data was normalised as described in the Materials and Methods and undetectable genes were removed. Differentially regulated transcripts were obtained by taking those that were at least 2-fold up- or downregulated in infected samples vs. controls at any time-point, and those that were significantly different by two-way ANOVA $p<0.05$ with Benjamini Hochberg multi-test correction. This left 396 transcripts, which were subjected to hierarchical clustering using Pearson centred distance metric and complete linkage. Normalised expression was visualised using a heat map.

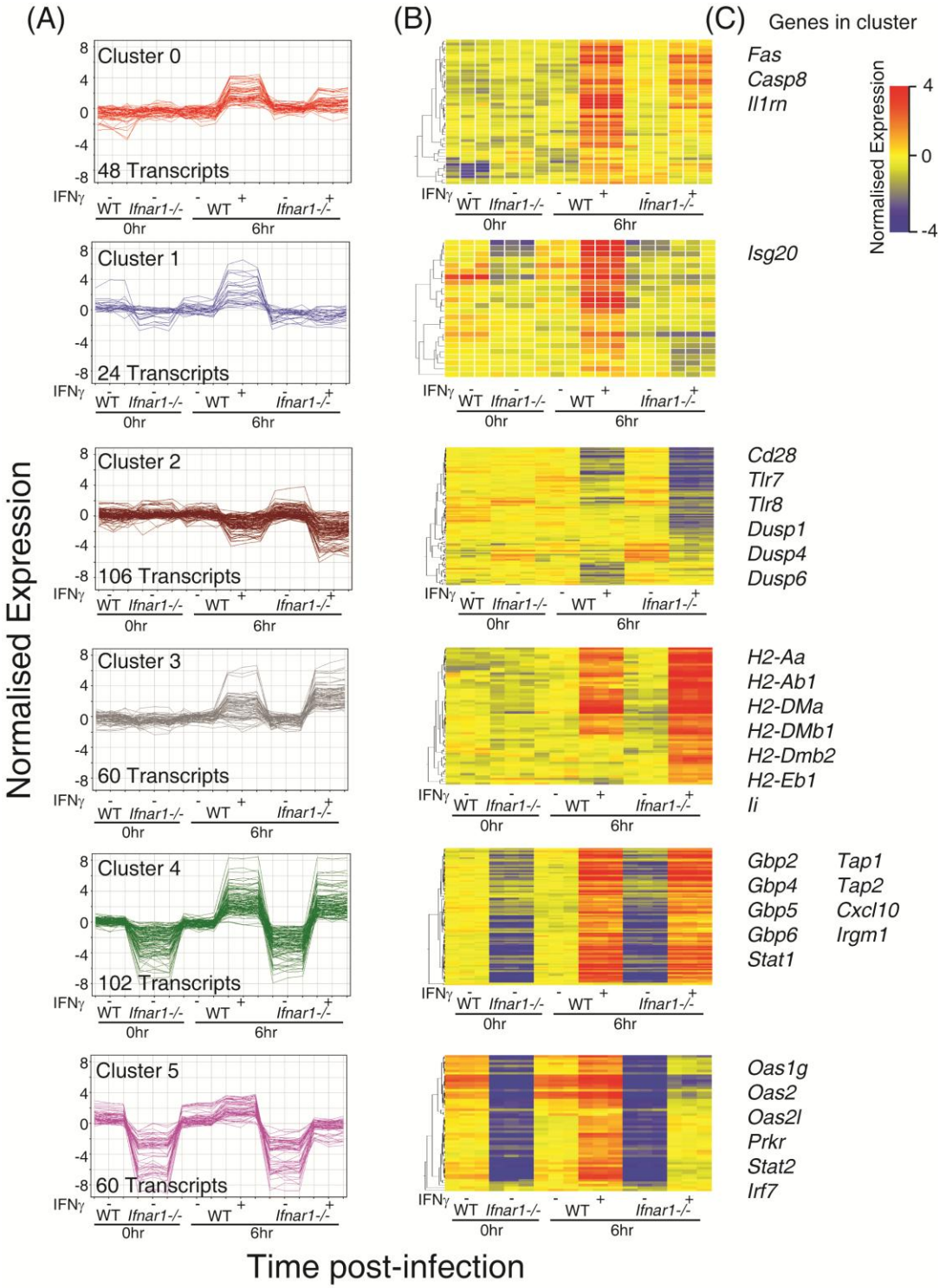

Figure 6.5. Clusters of genes are differentially regulated by type I IFN following IFNγ stimulation of macrophages. The 396 differentially regulated transcripts shown in Figure 6.4 were subjected to *k*-means clustering using Pearson centred distance metric, to generate 6 clusters. (A) Expression profiles of the six clusters. (B) The genes in each cluster were clustered hierarchically and visualised as a heat map.

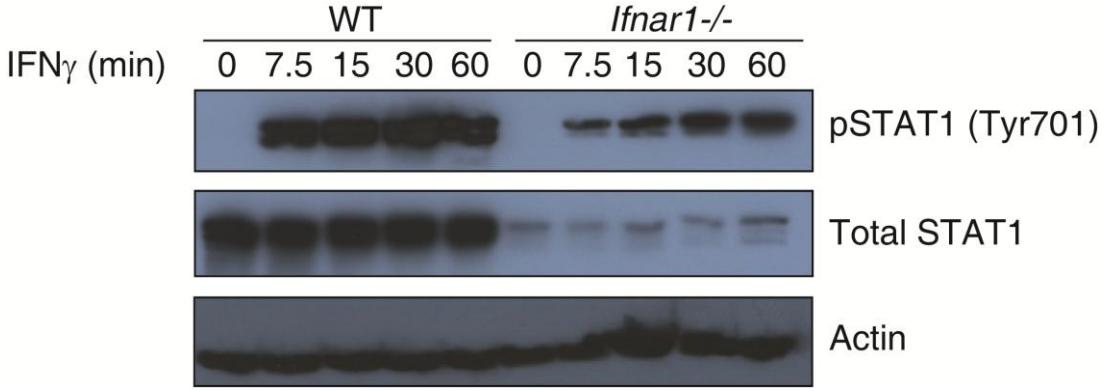

Figure 6.6. STAT1 phosphorylation by IFNγ is impaired in the absence of endogenous type I IFN. WT and *Ifnar1*$^{-/-}$ macrophages were stimulated with 5ng/ml IFNγ and whole cell extracts were prepared at the indicated time post-stimulation. Extracts were analysed by immuno-blotting using the antibodies shown. Data is from one experiment.

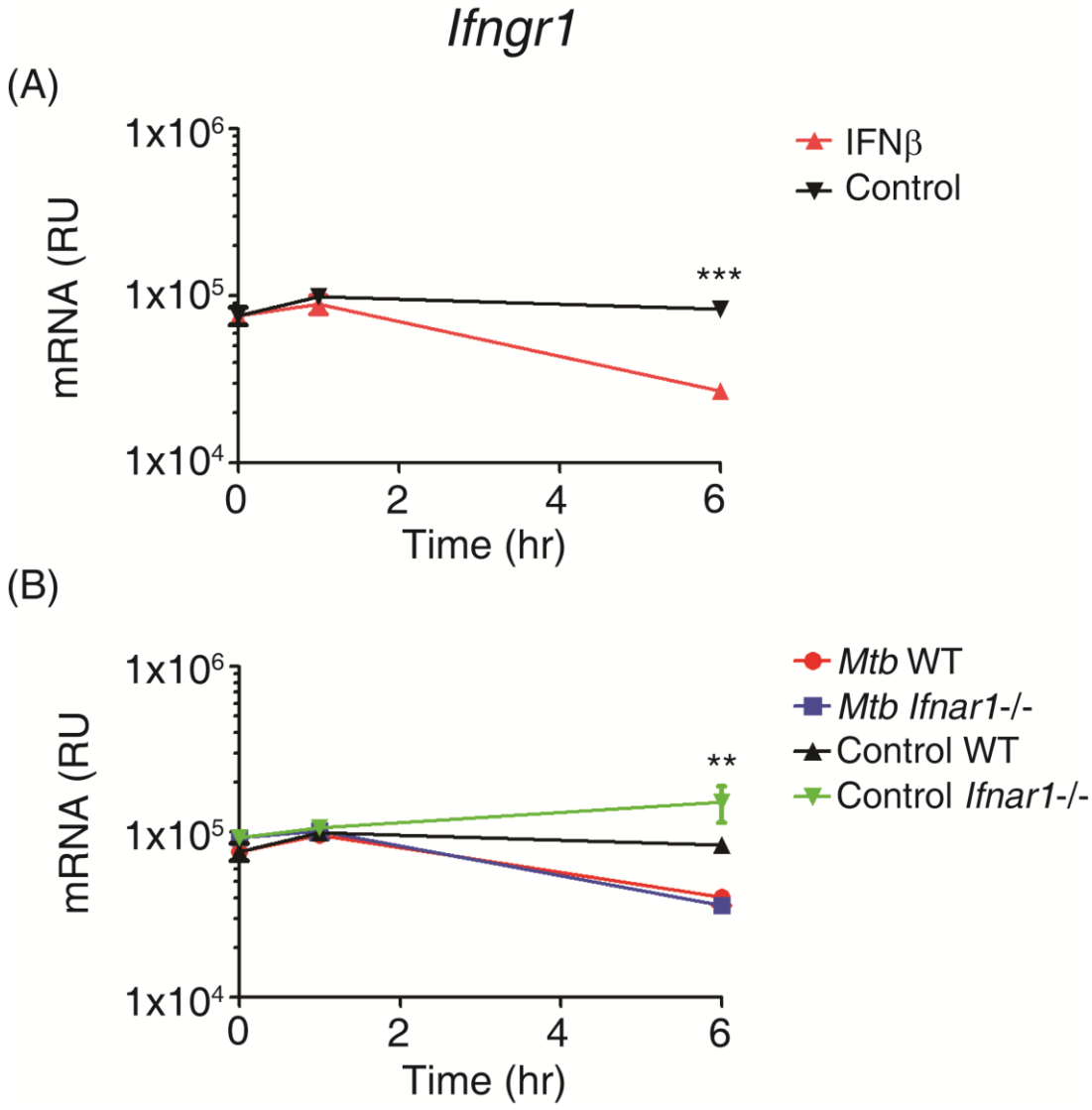

Figure 6.7. Type I IFN can regulate expression of the IFNγ receptor. (A) WT macrophages were treated with IFNβ at 2ng/ml, RNA was harvested at 0hr, 1hr and 6hr, converted to cDNA and *Ifngr1* mRNA levels determined by qPCR. (B) WT and *Ifnar1*$^{-/-}$ macrophages were infected with *Mtb*. RNA was harvested at 0hr, 1hr and 6hr, converted to cDNA and *Ifngr1* mRNA levels determined by qPCR. Graphs show mean +/- SEM (n=3) *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA with Bonferroni post-test. Data is from one experiment.

6.5. Transcripts present in six *k*-means clusters described in Figure 6.5

Cluster 0	
1110059G02Rik	Zranb3
4930422J18Rik	
Adora2b	
Armcx6	
AY078069	
BC057079	
Brdt	
Bzw2	
Casp8	
Casp8	
Ccnd1	
Ccnd1	
Ccnd1	
Ccrn4l	
Csrp1	
Cxcl11	
Cxcl2	
Dmwd	
Elmo1	
Fas	
Furin	
Gadd45g	
Gadd45g	
Hmgn3	
Hmgn3	
Hspa2	
Hspa2	
Il18rap	
Il1rn	
Itpkb	
Klrk1	
Laptm4b	
LOC224532	
LOC240672	
LOC278097	
Mgl2	
Nup93	
Palm	
Ppa1	
Psip1	
Rhoj	
Slc24a3	
Ssbp2	
Timp1	
Tmpo	
Tnfrsf12a	
Vwf	

Cluster 1
1700027J05Rik
2310016C08Rik
Akap12
Ccl3
D030012E24Rik
D030012E24Rik
D630022O22Rik
Dgat2
I830077J02Rik
Isg20
LOC223672
LOC270152
Mmp13
Mmp13
Ogfrl1
Paox
Paox
Pdk3
Pou3f1
Rin2
Rin2
Siat10
Socs2
Uaca

Cluster 2		
1110025F24Rik	Fvt1	St6gal1
1110032E23Rik	Garnl3	Stk17b
1300010M03Rik	Gdf15	Thbs1
1500010J02Rik	Gtf2ird2	Tlr13
1700023M03Rik	Hdac5	Tlr7
1810027I20Rik	Hlx1	Tlr8
2810408E11Rik	Hvcn1	Tnfrsf22
2810416G20Rik	Ihpk1	Trim25
3830612M24	Kif23	Uhrf1
4933411B03Rik	LOC329357	Ung
6330406L22Rik	LOC381484	Yaf2
6430511F03	LOC383435	Zdhhc14
6720463M24Rik	Lpxn	
A930001D11Rik	Lyl1	
AA408296	Mbd5	
Adam8	Mcm6	
Ahnak	Mcm6	
Ankrd47	Mgst2	
Apobec1	Mospd1	
Arhgap18	Mospd1	
B930045J24Rik	Mospd1	
Bach2	Msrb2	
BC006933	Msrb2	
Bcl2l11	Mtus1	
Birc1e	Necap1	
Cbr3	Necap1	
Cd28	Necap1	
Cd28	Numa1	
Cd28	Olfml2b	
Cerk	P2ry5	
Chaf1b	Pank1	
Clec4a3	Pde2a	
Clspn	Pparg	
Coq7	Ppp2r5d	
Cxcr4	Pscdbp	
Ddef1	Ptpn8	
Dgkz	Rasa3	
Dleu2	Rassf2	
Dscr1	Rbm38	
Dusp1	Rcbtb2	
Dusp4	Ris2	
Dusp6	Selenbp1	
Ebi2	Setmar	
Ehd4	Sgol1	
Epb4.1l1	Slc9a3r1	
Epb4.1l1	Sox4	
Eya1	Srpk2	

Cluster 3	
1300017K07Rik	Pvrl2
1810008K03Rik	Pvrl2
2210421G13Rik	Rarres1
5730528L13Rik	Rasgef1b
9130211I03Rik	Sdccag33
Acsl1	Synpo
Asns	Tbc1d2
C230021P08Rik	Tgfb1i4
Card4	Tnfrsf5
Ccl24	Vdr
Cd40	Vdr
Clec4n	Ybx3
Clec5a	Zhx2
Clec5a	
Clec9a	
Clecsf12	
Clecsf12	
Crsp2	
Ednrb	
F2rl1	
Fscn1	
Gbp2	
Gca	
Gja4	
H2-Aa	
H2-Ab1	
H2-Ab1	
H2-Ab1	
H2-Ab1	
H2-DMa	
H2-DMa	
H2-DMb1	
H2-DMb2	
H2-Eb1	
H2-M2	
Hivep3	
Igf2bp3	
Ii	
Ikzf1	
Kit	
Klf7	
Klhl6	
Klhl6	
Lass6	
LOC193676	
Pdlim1	
Prdx5	

Cluster 4		
2010106G01Rik	Gbp6	Stat1
2600010E01Rik	Glipr2	Stat1
4933430F08Rik	Glipr2	Tap1
5133400O11Rik	Gsdmdc1	Tap2
5830484A20Rik	Gvin1	Tapbpl
9930111J21Rik	H2-Q5	Trafd1
A130015P11Rik	H2-Q7	Trafd1
A330042I21Rik	H2-T23	Whdc1
A530060O05Rik	Ifi204	
A630077B13Rik	Ifi205	
A630085K21	Ifi205	
AA467197	Ifi205	
AI451557	Ifi205	
AI481100	Ifi47	
Aif1	Igtp	
BC006779	Ii	
BC006779	Ii	
BC013712	Irg1	
BC023741	Irg1	
BC049975	Irgm	
C3	Irgm	
Casp4	LOC215405	
Ccl12	LOC240327	
Ccl12	LOC435565	
Ccl5	LOC620078	
Ccl7	Mlkl	
Ccl8	Nupr1	
Cd69	Parp14	
Cdkn1a	Pdcd1lg1	
Clcn7	Psmb10	
Cp	Psmb8	
Cxcl10	Psmb8	
Cxcl10	Psmb9	
D12Ertd647e	Psme1	
D12Ertd647e	Ptafr	
Dlm1-pending	Rasgrp1	
F730045P10Rik	Rhoe	
F830008K13Rik	Rnase6	
Fcrl3	Samhd1	
Fpr-rs2	Samhd1	
Gadd45b	Samhd1	
Gbp2	scl000868.1_2	
Gbp2	scl000868.1_2	
Gbp4	Serpina3g	
Gbp4	Slamf7	
Gbp5	Slco3a1	
Gbp6	Spn	

Cluster 5	
2510004L01Rik	Sp100
Ass1	Stat2
D11Ertd759e	Tor3a
D11Lgp2e	Trex1
D11Lgp2e	Trex1
D11Lgp2e	Tyki
D14Ertd668e	Ube2l6
D14Ertd668e	Ube2l6
Ddit3	Usp18
Fcgr1	Usp18
G1p2	Usp18
H2-Q5	
H2-Q8	
H2-T23	
H2-T23	
H2-T9	
Ifi35	
Ifit2	
Ifit2	
Ifit3	
Ifitm1	
Ifitm3	
Irf7	
Isgf3g	
Isgf3g	
Lgals3bp	
Lgals9	
LOC380706	
LOC381276	
Luzp1	
Ly6a	
Ly6e	
Ly6e	
Oas1g	
Oas1g	
Oas2	
Oasl2	
Oasl2	
Olfr538	
Phf11	
Phf11	
Prkr	
Rnf34	
Sp100	
Sp100	

Table 6.1. Complete list of genes in six *k*-means clusters shown in Figure 6.5

Chapter 7. Summary and future perspectives

7.1. Summary

In this study I have carried out a comprehensive analysis of the transcriptional response of macrophages to *Mtb* infection over time. The results have illustrated the complexity of *Mtb*-macrophage interactions, with clusters of genes induced with different kinetics after infection. Two clusters were upregulated rapidly in *Mtb*-infected macrophages (30mins-1hr post-infection); one containing many cytokines and chemokines, and a second containing several genes regulated by the TPL2-ERK pathway. Clusters upregulated at later timepoints contained genes involved in MHC class I antigen presentation, steroid biosynthesis, apoptosis and type I IFN signalling. Many of these processes are likely to be important in resisting *Mtb* infection, and activating the adaptive immune response. However, the induction of genes involved in steroid biosynthesis may represent a virulence strategy of *Mtb*, allowing it to survive and replicate within macrophages.

The results of this initial microarray study prompted us to further investigate the role of two signalling pathways in the macrophage response; type I IFN and TPL2-ERK. Both pathways were found to regulate the production of important cytokines in response to *Mtb* infection. The TPL2-ERK pathway negative regulated the production of type I IFN, which indirectly enhanced the production of IL-1β, illustrating that early activation of the TPL2-ERK pathway can regulate the later macrophage response. The TPL2-ERK pathway also promoted the production of TNFα from *Mtb* infected macrophages, but inhibited the production of IL-12p40 and IL-12p70. Exogenous type I IFN enhanced the production of the immunosuppressive cytokine IL-10, which led to a suppression of IL-12p40 and TNFα, and also suppressed IL-1β independently of IL-10. In addition, microarray analysis of *Mtb*-

infected macrophages deficient in *Tpl2* or *Ifnar1* showed that both type I IFN and TPL2-ERK signalling were important regulators of transcription in response to *Mtb*.

Finally, we found that type I IFN could dramatically affect the macrophage response to IFNγ, both transcriptionally and functionally. Most importantly, type I IFN inhibited IFNγ-mediated upregulation of IL-12 production, and killing of *Mtb*, in macrophages, processes which are crucial to the protective immune response. Type I IFN can therefore regulate the immune response to *Mtb* at a number of stages, which is likely to explain the observation that mice deficient in type I IFN signalling mount a more effective immune response to *Mtb*.

7.2. Future perspectives

7.2.1. Transcriptional analysis of macrophages infected with diverse strains of *Mtb*

Our study was carried out using a single strain of *Mtb*, H37Rv. Globally, *Mtb* strains show a large amount of genetic diversity, with several distinct lineages associated with particular geographic areas (Gagneux and Small, 2007). A number of studies have reported that *Mtb* strains differ dramatically in their virulence, a good example being the hypervirulent W-Beijing lineage (Nicol and Wilkinson, 2008). Results have also suggested that interactions with the innate immune system may explain the hypervirulence of strains such as W-Beijing family members; for example Reed *et al* (2004) showed that presence of a lipid unique to W-Beijing family members, phenolic glycolipid (PGL), could actively downregulate the production of pro-inflammatory cytokines from macrophages.

A future direction would be to repeat the transcriptional analyses described in Chapter 3 with *Mtb* strains from several different lineages, including highly virulent

isolates such as members of the W-Beijing family. This is likely to reveal additional strain-specific virulence mechanisms possessed by highly virulent strains, and could lead to a better understanding of host-pathogen interactions.

7.2.2. Mechanistic studies into the regulation of cytokines by type I IFN

We found that both endogenous and exogenous type I IFN could regulate cytokine production from *Mtb*-infected macrophages. In some cases, we uncovered the mechanism behind these effects; for example, the suppression of IL-12p40 and TNFα by exogenous IFNβ was shown to be largely indirect, through the enhancement of IL-10 production. However, further research is required to understand the mechanisms behind other effects of type I IFN which were IL-10 independent.

Given that IL-10 underscores a number of effects of type I IFN, how type I IFN regulates IL-10 is of particular interest. A number of mechanisms have been put forward by other studies, for example IL-27 was suggested as an indirect mechanism for the enhancement of IL-10 by type I IFN (Iyer *et al*, 2010). This will be investigated in our system by using mice deficient in the IL-27 receptor component WSX-1. The effect of type I IFN on IL-10 appears to be transcriptional, as IFNβ led to a prolonged upregulation of IL-10 mRNA production in *Mtb*-infected macrophages. Thus, a potential avenue of enquiry would be to determine transcription factor binding at the IL-10 promoter; in particular the binding of type I IFN inducible transcription factors such as STAT1. The IL-10 promoter has been reported to contain STAT1 binding sites (Saraiva and O'Garra, 2010), suggesting this may present a potential mechanism. A further possibility is regulation of IL-10

mRNA stability by type I IFN. This is suggested by our findings showing that addition of IFNβ leads to sustained IL-10 mRNA levels, which could reflect increased stability as opposed to an enhanced transcription. IL-10 mRNA is known to be regulated at the level of stability by factors such as tristetraprolin (TTP) (Stoecklin *et al*, 2008) and type I IFN could be involved in regulating this process.

We found that addition of IFNβ led to suppression of IL-1β at the transcriptional level. However, Guarda *et al* (2011) have shown that IFNβ could also regulate inflammasome activation in response to stimulation with LPS and alum, representing a further mechanism by which type I IFN could inhibit IL-1β. Future work could therefore determine if inflammasome activation by *Mtb* is inhibited by type I IFN.

7.2.3. Regulation of immune processes by type I IFN in additional cell types, and *in vivo*, in response to *Mtb* infection

In this study we investigated the response to *Mtb* in bone-marrow derived macrophages, as macrophages are a major host-cell for *Mtb in vivo* (Wolf *et al*, 2007) and are thought to mediate the majority of *Mtb* killing (Flynn and Chan, 2001). However, a number of other innate immune cells can become infected during infection, such as DCs and neutrophils (Wolf *et al*, 2007; Eum *et al*, 2009) and these cells can also produce cytokines in response to triggering of PRRs by *Mtb*. Future work could therefore address whether the pathways we describe here also regulate cytokine production in additional cell types.

The major producers of IL-12 during *in vivo Mtb* infection are thought to be myeloid DCs (Rothfuchs *et al*, 2009). It would therefore be of interest to determine if IFNβ also suppresses production from myeloid DCs infected with *Mtb*. The role of

the TPL2-ERK pathway in suppressing IL-12 production could also be investigated in this cell type. This could be addressed using bone-marrow derived dendritic cells as a model. Findings in bone marrow derived dendritic cells could then be validated by analysing DC expression of IL-12 *in vivo* during *Mtb* infection.

IL-10 can be made by many cell types besides macrophages, including innate cells such as DCs and neutrophils, and adaptive immune cells such as T cells and B cells (Saraiva and O'Garra, 2010). Thus, whether type I IFN promotes IL-10 production in a wide range of immune cells, or specifically in macrophages, would be of interest. To address this, others in our laboratory are making use of 10BiT mice, IL-10 reporter mice in which cells expressing IL-10 upregulate Thy1.1 on the cell surface, which can be detected by flow cytometry (Maynard *et al*, 2007). We have crossed these mice with *Ifnar1*$^{-/-}$ mice, and can thus address the role of type I IFN in promoting IL-10 production *in vivo* during *Mtb* infection, by comparing Thy1.1 expression in 10BiT WT mice with 10BiT *Ifnar1*$^{-/-}$ mice. This strategy can thus be used to determine IL-10 production in a variety of immune cells during *Mtb* infection.

Of particular interest is IL-10 production by neutrophils, as a recent study reported that neutrophils produced high levels of IL-10 *in vitro* in response to BCG and *Mtb* (Zhang *et al*, 2009) and IL-10 producing neutrophils have been isolated from patients with melanoma (De Santo *et al*, 2011). Given that the IFN signature described by Berry *et al* (2010) is largely found within neutrophils in the blood, type I IFN could potentially be driving IL-10 production from neutrophils.

7.2.4. Confirmation of microarray results by functional analyses

Although microarray is a powerful tool for assessing the response of cells to stimulation or infection, a drawback of this technique is that it operates purely at the level of transcription. This is a caveat because gene expression is extensively regulated post-transcriptionally, through mechanisms such as mRNA stability, and covalent modifications such as phosphorylation and ubiquitination. Thus, a change in gene expression at the mRNA level may not correspond to increased protein production. We found that type I IFN could affect the expression of genes involved in a wide range of processes, besides cytokine production. For example, a number of apoptosis related genes were regulated by type I IFN and IFNγ. In addition, the induction of genes involved in the MHC class II antigen presentation pathway, such as MHC class II molecules and the invariant chain Ii, by IFNγ was inhibited by type I IFN. It is important therefore that these effects are confirmed at the protein level and by functional analyses. For example, apoptosis could be assessed by a number of different assays, such as TUNNEL staining, and antigen presentation could be assayed by incubating peptide-pulsed WT or *Ifnar1*$^{-/-}$ DCs with TCR-transgenic naive T cells as a pool, and determining subsequent T cell proliferation or cytokine production.

In addition to apoptosis and antigen-presentation associated genes, microarray analysis also revealed that type I IFN regulated production of iNOS, which is critical for killing of *Mtb* in macrophages (Chan *et al*, 1992), and the production of several chemokines such as *Ccl2* and *Ccl7*, which are required for migration of monocytes to the *Mtb* infected lung (Peters *et al*, 2000). Regulation of iNOS could be confirmed by using Greiss's reagent to determine the amount of nitric oxide in macrophages by spectrophotometry, as used by Ehrt *et al* (2001). The

regulation of chemokines by type I IFN could be determined either by ELISA, or by a more global multiplex analysis. This approach has the advantage of revealing potential post-transcriptional regulation of cytokines and chemokines by type I IFN.

7.3. Conclusion

This study reveals important signalling events that occur at different times following *Mtb* infection of macrophages, which are likely to have an impact on the outcome of *Mtb* infection *in vivo*.

Chapter 8. References

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